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The Nature of Memory $CD8^+$ T-Cell Responses in Bovine Tuberculosis

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Abstract

M. bovis is a zoonosis and is the aetiological agent of bovine TB. The incidence of bovine TB in the UK is increasing, demonstrating that the current test and slaughter policy is failing. *M. bovis*-BCG, the vaccine used against human TB, is not used in cattle as it interferes with the tuberculin skin test and only confers partial protection. The control of bovine TB will require the design of a more efficacious vaccine and discriminatory diagnostic test. To achieve this, a greater understanding of both the immune response induced by the mycobacteria and the protective immune response required to clear or control *M. bovis* infection in cattle is needed. In human and mouse studies, $CD8^+$ T cells have been proposed to play an important role in immunity to mycobacteria. The aim of this study was to identify and define the $CD8^+$ T cells induced by BCG vaccination and/or *M. bovis* infection in cattle.

Initial experiments identified different subsets of $CD8^+$ T cells present in cattle using expression of surface and effector molecules. The $CD8^+$ population is heterogeneous and contains αβ ($TCR1^-CD3^+$), γδ ($TCR1^+CD3^+$) and NK ($TCR1^-CD3^-$) cells. $CD8^+$ cells could be divided into $CD8^{hi}CD3^+$ and $CD8^{lo}CD3^{+/-}$. The ($CD8^{hi}TCR1^-$) $\alpha\beta^+$ T cells were analysed in this study as these form part of the adaptive immune response. Age-related increases in the percentage of activated/memory $CD8^{hi}TCR1^-$ T cells were observed in blood as shown by increases in expression of the activation marker CD25 and memory marker CD45RO. Concomitant decreases were observed in the percentage of $CD8^{hi}TCR1^-$T cells that express CD45RA, CD62L and CD27, surface molecules associated with naïve T cells. The percentage of $CD8^{hi}TCR1^-$ T cells in blood expressing perforin and IFN-γ also increased with age. The $CD8^{hi}TCR1^-$T cells present in the BAL were mainly activated/memory T cells as shown by a combination of expression of surface and effector molecules. In contrast, the $CD8^{hi}TCR1^-$T cells present in the lymph nodes more resembled naïve T cells with only a small percentage expressing perforin and IFN-γ. A minor population of $CD8^{hi}TCR1^-CD45RO^+CD62L^+$ T cells were present in the LN which may be the bovine equivalent of murine central memory cells.

BCG vaccination of cattle induces mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells. These cells mediated recall responses to BCG, detected by proliferation, production of IFN-γ, up-regulation of perforin expression and lysis of BCG-infected macrophages (Mϕ). *M. bovis* reactive-$CD8^{hi}TCR1^-CD45RO^+$ T cells were detected in non-vaccinated and BCG-vaccinated animals after infection with *M. bovis*. These cells proliferated and produced IFN-γ in response to *M. bovis*-infected but not BCG-infected Mϕ, indicating that they were responding to an *M. bovis*-specific antigen. Post-challenge responses in the $CD8^{hi}TCR1^-CD45RO^+$ T cells from BCG-vaccinees were considerably greater than those of non-vaccinated animals. *M. bovis*-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells reduced mycobacterial viability when cultured with BCG- or *M. bovis*-infected Mϕ. $CD8^{hi}TCR1^-CD45RO^+$ T cells present in the lungs of *M. bovis*-infected animals expressed IFN-γ after culture with mycobacteria. These results demonstrate that mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells that express effector functions are present at the site of infection, and may contribute to the control of *M. bovis* infection in cattle. This study provides a basis to investigate the role of $CD8^{hi}TCR1^-CD45RO^+$ T cells in immunity to *M. bovis* infection in cattle and to evaluate the effectiveness of new TB vaccines at inducing $CD8^+$ T cell responses.

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Declaration

I, Alison Elizabeth Hogg, confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abbreviations

2-ME	2-mercaptoethanol
AIDS	Acquired immune deficiency syndrome
Ag85	Antigen 85 complex
APC	Antigen presenting cell
ATP	Adenosine triphosphate
β-2m	Beta-2 microglobulin
BAL	Bronchoalveolar lavage
BCG	*Mycobacterium bovis* bacillus Camette Guerin
bp	Base pair
cDNA	Complementary DNA
^0C	Degrees Celsius
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CFP	Culture filtrate protein
CO_2	Carbon dioxide
cfu	Colony forming units
CR	Complement receptor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecules-3 grabbing non-integrin
DMEM	Dulbeccos modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxyribonucleoside-5'-triphosphate
DTH	Delayed type hypersensitivity reaction
EDTA	Ethylene diamine tetra acetic acid
ER	Endoplasmic reticulum
ELISA	Enzyme-linked immunoabsorbant assay
ESAT-6	Early secreted antigenic target 6
FACS	Fluorescence activated cell sorter
Fc R	Fc receptor
FCS	Foetal calf serum
FITC	Fluroscein isothyocyanate
FSC	Forward scatter
GM-CSF	Granulocyte/macrophage colony stimulating factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
H_2O	Water
HRP	Horseradish peroxidase
HSP	Heat shock protein
IAH	Institute for Animal Health
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL-	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1R-associated kinase
kDa	KiloDaltons
KO	Knock-out
LAM	Lipoarabinomannan

LAMP	Lysosome-associated membrane glycoprotein
LM	Lipomannans
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
ManLAM	Mannose-capped lipoarabinomannan
MAP	Mitogen activated protein
MBSE	*Mycobacterium bovis* sonic extract
MCP-1	Monocyte chemoattractant protein 1
Mϕ	Macrophage
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mth	Month
MR	Mannose receptor
mRNA	Messenger ribonucleic acid
MDR	Multi-drug resistant
MVA	Modified vaccinia Ankara strain
MW	Molecular weight
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
Nramp1	Natural resistance associated macrophage protein
OD	Optical density
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBSa	Phosphate buffered saline
PCR	Polymerase chain reaction
PPD-A	Purified protein derivative from *M. avium*
PPD-B	Purified protein derivative from *M. bovis*
PRR	Pattern recognition receptors
RANTES	Regulated on activation normal T cell expressed and secreted
RD	Region of difference
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
RPE	R-phycoerythrin
RPMI	Roswell park memorial institute
RT-PCR	Reverse transcriptase PCR
SSC	Side scatter
Sp-A	Surfactant protein A
TACO	Tryptophan-aspartate containing coat protein
TAK1	TGF-β activated kinase 1
TAP	Transporter associated with antigen presentation
TB	Tuberculosis
T-cell	T-lymphocyte
TCM	Tissue culture medium
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Th1	T helper 1
TLR	Toll-like receptor

TNF-α	Tumour necrosis factor-alpha
TRAF	Tumour necrosis factor receptor associated factor
UK	United kingdom
VV	Vaccinia virus
WHO	World health organisation
wt	Wild type
yr	Year

CHAPTER 1: INTRODUCTION

1.1 Mycobacteria

1.1.1 General characteristics

Mycobacteria are aerobic, unicellular, non-spore-forming gram-positive bacteria. Taxonomically, mycobacteria belong to the genus *Mycobacterium*, forming a monophyletic taxon within the family of Actinomycetes (Embley and Stackebrandt 1994). Unlike other gram-positive bacteria, mycobacteria do not retain the gram stain due to the unique hydrophobic structure of the mycobacterial cell wall. Mycobacteria are therefore identified as being acid-fast (retain basic dyes in the presence of acid alcohol) and are detected by the use of the Ziehl-Nielsen stain (Taracha, Bishop et al. 2003; Ulrichs, Lefmann et al. 2005).

The genus Mycobacterium is comprised of 85 different strains and the majority of these are non-pathogenic environmental bacteria closely related to the soil bacteria Streptomyces and Actinomyces. However, a few species are highly pathogenic which include some of those defined in the *M. tuberculosis* complex. The *M. tuberculosis* complex consists of: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canettii* and *M. microti*.

The advent of high-throughput sequencing technology has facilitated the sequencing of the genomes of *M. tuberculosis* (H37rV and CDC1551) and *M. bovis* (AF2122/97) and revealed that at the nucleotide level the two genomes share >99.95% identity (Garnier, Eiglmeier et al. 2003). Compared to the *M. tuberculosis* genome which encodes 3995 proteins, the *M. bovis* genome encodes 3951, contains 11 deleted regions (RD) and only one unique locus TbD1(Garnier, Eiglmeier et al. 2003). These findings suggest that *M. bovis* has undergone a genome down-sizing. It was previously thought that *M. tuberculosis*, of which humans are the only natural host, may have evolved from *M. bovis* which has a broad host range including humans and cattle. However, the deletion of genes from *M. bovis* compared to *M. tuberculosis* suggests that this may not be the case and that members of the *M. tuberculosis* complex may have in fact evolved from a common ancestor (Brosch, Gordon et al. 2002). It has been proposed that *M. tuberculosis* is the most ancestral member of the *M. tuberculosis* complex and that

through the loss of RD regions in different combinations the various species, sub-species and strains have been generated (Mostowy, Cousins et al. 2002).

1.2 Epidemiology of Tuberculosis

1.2.1 Epidemiology of Bovine TB

Bovine TB is a major economic concern in Great Britain (GB) as the incidence of disease in cattle herds has been increasing at an average of 20% per year since the early 1990s. (Krebs and Group 1997; Reynolds 2006). In 2004 the extent of the problem was demonstrated with approximately 99,000 registered herds being under movement restrictions, accounting for approximately 5.6% of the national herd (Reynolds 2006). Worryingly disease hotspots have been identified in the UK wherein higher percentages of herds are under TB restriction, these areas include Cornwall (15%) and Gloucestershire (25%). Control of bovine TB relies on the regular testing of herds using the intradermal tuberculin skin test and the subsequent slaughter of animals that test positive. In 2002 approximately 22 000 animals were slaughtered, costing the government in the UK approximately £31 million, by 2005 these costs had risen to over £90 million.

In developing countries, where the incidence of bovine TB is thought to be high, there are no control measures against bovine TB. Therefore transmission rates of *M. bovis* from infected cattle to humans are also likely to be high which further substantiates the need for improved inexpensive control measures that can be used in both the UK and developing countries.

The presence of a wildlife reservoir of *M. bovis* has been proposed as an important contributory factor to the increasing incidence of bovine TB in UK. Owing to its broad host range, *M. bovis* infects numerous wildlife species including badgers, deer and red foxes (Delahay, De Leeuw et al. 2002). Of particular relevance is the reservoir of *M. bovis* in the european badger *Meles meles* which has been proposed to contribute significantly to the increasing incidence of bovine TB in the UK. Widespread culling of possums, the main wildlife reservoir of *M. bovis* infection in New Zealand led to a reduction in the incidence of *M. bovis* infection in cattle herds. However culling trials of badgers in the UK have provided variable results (Mairtin, Williams et al. 1998;

Donnelly, Woodroffe et al. 2003). The most recent trial showed that the incidence of *M. bovis* infection in cattle declined in the culled areas but infection rates significantly increased in the surrounding areas (Donnelly, Woodroffe et al. 2003). A vaccination trial of badgers will begin soon in the UK which may provide a more effective and humane method of eradication of *M. bovis* aside from larger scale culling trials.

Although *M. bovis* and *M. tuberculosis* are the primary causes of bovine and human TB respectively, these two mycobacteria are genetically very similar but have different host ranges. *M. bovis* has a broad host range and causes disease in both humans and cattle whereas *M. tuberculosis* is primarily a human pathogen and does not cause disease in cattle (O'Reilly and Daborn 1995; de Lisle, Mackintosh et al. 2001).
In the UK, prior to pasteurisation of milk, ingested *M. bovis* was the primary cause of non-pulmonary TB in humans with an annual rate of 50 000 new cases. The disease typically affected the cervical lymph nodes, intestinal tract, meninges and in rare cases the skin (Wilkins, Griffiths et al. 1986) In addition, *M. bovis* can also be contracted by humans through the respiratory tract via contact with infected cattle resulting in the development of typical pulmonary TB. The pasteurisation of milk and implementation of BCG vaccination significantly reduced the incidence of *M. bovis* infections in humans in the UK. However, even in countries with low incidence rates of bovine TB a small number of cases still arise due to the reactivation of dormant infections in the elderly (Grange and Yates 1994).
At present *M. bovis* infection in humans is thought to account for as many as 5-10% of recorded TB infections (O'Reilly and Daborn 1995; Cosivi, Grange et al. 1998). Most of these cases occur in developing countries where there no controls strategies in place. Moreover, in these countries the incidence of HIV and AIDS is also high with mycobacterial infections being one of the most common opportunistic infections affecting immunosuppressed HIV patients (Grange and Yates 1994).

1.2.2 Epidemiology of Human TB

Tuberculosis in humans is primarily caused by *M. tuberculosis* and is one of the leading causes of death worldwide. Since its formal identification by Robert Koch 123 years ago, the understanding of disease pathogenesis has increased substantially, however the prevalance of tuberculosis is ever increasing. TB kills nearly 3 million people annually

with an estimated one third of the worlds population being infected with *M. tuberculosis* and 8 million new cases each year, this figure is likely to rise. The World Health Organisation (WHO) estimates that between 2000 to 2020, the number of people infected with *M. tuberculosis* is likely to double; in this time 200 million people will develop TB and 35 million will die from the disease (Dye, Scheele et al. 1999). The majority of cases occur in developing countries with sub-sahara Africa having the highest incidence rate per capita and Southeast Asia showing the highest number of reported cases (Corbett, Watt et al. 2003).

The increasing prevalance of TB is fuelled by the HIV/AIDS pandemic as HIV/TB co-infection is the highest risk factor for disease progression (Corbett, Watt et al. 2003). It is thought that greater than one third of HIV-patients are co-infected with *M. tuberculosis* and 12 % of AIDS deaths have been attributed to TB (Espinal, Dye et al. 1999; Corbett 2003; Corbett, Watt et al. 2003).

In immunocompetent individuals that become infected with *M. tuberculosis*, only 5-10% of those will develop active disease within 1-5 yrs post-infection (Comstock 1982). The remainder will control but not clear the infection and are said to be latently infected and have a 5-10% lifelong risk of developing active disease. Latent infection occurs when a state of equilibrium is established between an individual's immune system and the mycobacteria resulting in containment of infection. The risk of developing active clinical TB is substantially increased in individuals who are co-infected with HIV and *M. tuberculosis* as these individuals have a 10% annual risk of developing disease.

In the last 50 years, the problem of multidrug-resistant TB (MDR-TB) has arisen and is thought to be a man-made epidemic due to patients failing to complete the 6 mth antibiotic therapy. These strains of TB do not respond to the two most powerful anti-tuberculosis drugs available, isoniazid and rifampin. The incidence of MDR-TB is increasing, with as many as 50 million people infected; MDR-TB has been reported in more than 100 countries or territories. Poorly managed TB control programmes are thought to be the source of MDR-TB. Increased incidences of MDR-TB have been identified in some areas defined as MDR-TB hotspots these are Latvia, Delhi State of India, Estonia, Henan Province in China, Dominican Republic, Argentina, Russia, and the Ivory Coast (http://www.who.int/mediacentre/factsheets/fs104/en/index.html). The increasing incidence of MDR-TB further substantiates the urgent need for effective therapeutic and prophylactic vaccines.

1.3 Pathogenesis of TB

1.3.1 Pathogenesis of Bovine TB

The pathogenesis of TB in cattle is not as well defined as the pathogenesis of TB in humans but it is thought that the two are more similar than that observed in mouse models of TB. It is thought that *M. bovis* infection of cattle occurs mainly via the oral or respiratory route. Cattle are likely to become orally infected from the nursing of calves by infected mothers or through the ingestion of faeces from *M. bovis* infected badgers (Neill, Cassidy et al. 1994). The respiratory route is thought to be the most common route of *M. bovis* infection in cattle as 67% of cattle with TB have lesions in the lung and pulmonary lymph nodes. TB lesions in the cranial lymph nodes are observed in 39% of infected animals while only 8% had lesions involving the mesenteric lymph nodes (Phillips, Foster et al. 2003). Most studies of naturally infected cattle have reported that the majority of lesions were found within the lung, pulmonary lymph node and cranial lymph nodes (Lepper and Pearson 1973). Although these studies indicate that the respiratory route of infection is the most common route due to the pattern of lesions, it is not always possible to deduce the route of infection from the lesion distribution. Cattle infected orally with *M. bovis* developed lesions in the lung, pulmonary lymph nodes and cranial lymph nodes with no involvement of intestinal tract or mesenteric lymph nodes (Palmer, Waters et al. 2004).

In the field, the dose and frequency of exposure to *M. bovis* in cattle is unknown but is likely to be variable and repeated. Studies in animals experimentally infected with *M. bovis* have shown that bacterial shedding is transient and few live bacilli can be detected in the nasal mucosa (McCorry, Whelan et al. 2005). This suggests that transmission from animal to animal may be limited in the field. However, it was recently demonstrated that 50% of cattle experimentally infected intratracheally with as low as 1 cfu of *M. bovis* developed disease and showed similar pattern of pathology as animals given 1000 cfu (Dean, Rhodes et al. 2005; Johnson, Dean et al. 2006). These results indicate that cattle in the field do not have to be exposed to large number of bacilli in order to become infected with *M. bovis*. In these studies it was shown that animals infected with *M. bovis*, regardless of the dose, could be detected using a

combination of the comparative skin test and IFN-γ BOVIGAM test (Dean, Rhodes et al. 2005; Johnson, Dean et al. 2006).

It is thought that experimental infection with *M. bovis* via the respiratory route with a low dose of *M. bovis* is most likely to mimic natural infection. Past studies have used intranasal, intratracheal, intratonsilar and aerosolised infection (Palmer, Whipple et al. 1999); (Buddle, de Lisle et al. 1995; Cassidy, Bryson et al. 1998). Each of these methods of inoculation induces disease pathology that is similar to that observed in animals naturally infected. However artefacts of each inoculation method are also observed with lesions occurring at sites that are not seen during natural infection.

1.3.2 Pathogenesis of Human TB

Primary infection with *M. tuberculosis*, similar to *M. bovis*, most commonly occurs after the inhalation of airborne droplets of tubercle bacilli. After uptake of the mycobacteria by alveolar Mϕ, several scenerios can occur. It is clear that not all individuals that are exposed to *M. tuberculosis* become infected therefore in some instances, *M. tuberculosis* is immediately killed by the Mϕ before it is able to establish infection. However, in those that become infected the mycobacteria escape the antimicrobial functions of the Mϕ and multiply. Thereafter the infected Mϕ either die or become activated and produce inflammatory mediators such as cytokines and chemokines. Either way, inflammation is initiated and other innate immune cells such as monocytes, NK cells and neutrophils are recruited into the lung forming the early stages of the granuloma. The infiltrating monocytes differentiate into Mϕ and ingest but are unable to kill the mycobacteria. Within two to three weeks after the initial infection the adaptive response develops and *M. tuberculosis*-specific T cells are recruited to the site of infection where they proliferate, produce cytokines that activate Mϕ and kill infected Mϕ. At this stage, the growth of the mycobacteria is inhibited by the effector functions of the hosts immune response and is contained within the granuloma. The infection is stabilised and this phase is defined as latency. However, if the immune response fails to control the infection, progression to active disease occurs and the mycobacteria replicate within liquefied caesous foci of the failing granuloma. Under these circumstances, cavity formation can occur and lead to the rupture of nearby

bronchi, allowing the mycobacteria to disseminate through the airways and access the environment through expectoration of live bacilli (Rook and Hernandez-Pando 1996).

Active TB occurs in approximately 5-10% of infected individuals due to either a failure of the host's immune response to contain the primary infection or to reactivation of disease in individuals latently infected due to a decline in immune function. TB can occur anywhere in the body, but as much as 85% of active TB cases present as pulmonary infection ranging from mild infiltration to chronic, cavitary and severely destructive disease. However, in milliary TB, the most serious disease manifestation, large numbers of mycobacteria disseminate throughout the body. Thus, disease localisation, severity and outcome of active TB is highly variable and will reflect the immune status of the host.

Susceptibility to developing active disease has also been described in immuno-comprised individuals caused either by infection with HIV or functional deficiencies due to polymorphisms in genes encoding cytokines and cytokine receptors, chemokine and chemokine receptors and major histocompatibility complex (MHC) molecules (Bothamley, Beck et al. 1989; Casanova and Abel 2002).

1.4 Vaccines against Tuberculosis

1.4.1 Vaccines against Bovine TB

In 1997, an independent report by Krebs recommended that the long-term control of bovine TB will require the development of cattle TB vaccine and an accompanying sensitive and specific diagnostic test (Krebs and Group 1997). The need for an effective vaccine against bovine TB was re-affirmed in a report by the House of Commons Environment, Food and Rural Affairs Committee (2004).

1.4.1.1 *Mycobacterium bovis* bacille Calmette-Guerin (BCG)

The only vaccine currently licensed for use against TB in humans is BCG and is one of the most widely used human vaccines in the world with an estimated 3 billion doses administered since the 1920s (Lugosi 1992). BCG was derived from *M. bovis* isolated from a cow with tuberculous mastitis by serial passage on glycerinated bile potato medium. The continued propagation of BCG in laboratories worldwide has given rise to a number of daughter strains: BCG glaxo, BCG Pastuer, BCG tokyo, BCG danish (Behr 2002). Each strain displays different antigenic profiles due to a differential expression of antigens (Wiker, Nagai et al. 1996).

Since 1919 BCG has been trialled in cattle and has shown variable efficacy at conferring protection against *M. bovis*. This variability may be in part due to differences in the experimental conditions of the trials including vaccine sub-strain, route of infection, dose of inoculum and age of animals. Nevertheless, in response to *M. bovis* infection, BCG-vaccinated animals develop fewer less severe lesions compared to non-vaccinated animals. Thus, demonstrating that the immune response induced by BCG is able to control but not clear the *M. bovis* infection. It has been proposed that pre-exposure of animals to environmental mycobacteria reduces the efficacy of BCG vaccination (Buddle, Wards et al. 2002). In contrast, other reports have shown that reactivity to environmental mycobacteria did not affect BCG efficacy and can provide a level of protection against *M. bovis* infection (Buddle, Wedlock et al. 2003; Hope, Thom et al. 2005). Therefore the capacity of BCG to protect pre-sensitised animals may depend on the level of exposure to environmental mycobacteria. In order to avoid pre-sensitisation due to environmental mycobacteria or early exposure to *M. bovis*, cattle should be vaccinated with BCG as neonates. It has been shown that BCG vaccination is more effective when administered to neonatal calves compared to older calves (Buddle, Wards et al. 2002; Buddle, Wedlock et al. 2003). Therefore a similar protocol to that used for humans in developing countries and the UK, in which newborns are vaccinated with BCG, may be introduced for cattle.

Although BCG does not provide complete protection in either humans or cattle, it remains the prototype vaccine against which all novel TB vaccines will be compared. To date, no novel single vaccines have provided a greater level of protection in cattle or humans than BCG.

Furthermore, the safety record and low production costs make BCG an attractive potential vaccine for cattle but the introduction of BCG and mostly likely any TB vaccine will also require the generation of new diagnostic tests.

The only vaccination strategy that has been shown to confer a greater level of protection than BCG involves administration of BCG in conjunction with another vaccine. The procedure of administering two different vaccines that express the same antigens at separate times is termed heterolgous prime-boost strategy. In the last ten years, heterologous prime-boost strategies have been considered for the following diseases; HIV, malaria and human TB, showing a level of success (Dunachie and Hill 2003; Moore and Hill 2004; McShane, Pathan et al. 2004) In cattle, several different vaccines have been tested in combination with BCG which include DNA vaccines, protein subunit vaccines and recombinant virus vectors.

1.4.1.2 DNA subunit vaccines

DNA vaccines consist of a plasmid containing one or more vaccine antigens under the control of a viral promoter enabling expression of these antigens. The plasmid backbone of the vaccine contains unmethylated CpG motifs which are short stretches of DNA sequence with potent immunostimulatory properties. DNA vaccines showed promise in studies of small animal models of TB, however these vaccines when used alone confer no protection in cattle (Skinner, Buddle et al. 2003; Chambers, Stagg et al. 2001; Chambers, Williams et al. 2001). A heterologous prime-boost strategy involving priming with DNA and boosting with protein antigen was trialled using the mycobacterial heat-shock protein 65 (hsp65). This strategy induced stronger Th1 responses compared to DNA alone but not to the same extent as BCG (Vordermeier, Lowrie et al. 2003). However, priming with DNA and boosting with the mycobacterial antigen MPB70 did not protect animals against *M. bovis* infection. In addition, a greater amount of pathology was observed in the vaccinated animals compared to the nonvaccinated animals (Wedlock, Skinner et al. 2003).

A more effective strategy involved priming of the immune response with a cocktail of DNA vaccines encoding the mycobacterial proteins HSP65, HSP70 and APA followed by boosting with BCG. This prime-boost protocol induced a level of protection superior to that observed with BCG alone and the order in which the vaccines were administered did not affect the enhancement of protection (Skinner, Buddle et al. 2003). Six

parameters are used to determine vaccine efficacy these are; severity of the lesions, number of tissues affected; number of animals with lesions, mean lung lesion score, mean LN lesion score and bacterial load. The heterologous prime boost strategy involving DNA and BCG described above protected animals in all six parameters whereas in this experiment with BCG only provided protection in two of these parameters (Skinner, Buddle et al. 2003).

1.4.1.3 Protein subunit vaccines

Protein subunit vaccines when administered on their own are unlikely to induce a strong Th1 type immune response without the addition of an adjuvant. In mice, it was shown that naked DNA vaccines can enhance the protection afforded by BCG and this enhancement was attributed to presence of unmethylated CpG motifs in the DNA (Freidag, Melton et al. 2000; Hogarth, Logan et al. 2006). On this basis synthetic oligonucleotides containing unmethylated CpG motifs (CpG-ODN) have been synthesised and used in conjunction with protein subunit vaccines as adjuvants. The culture filtrate proteins (CFP) of *M. bovis* and *M. tuberculosis* have been shown in small animal models to be protective (Bosio and Orme 1998). In cattle, vaccination trials of CFP administered in combination with different adjuvants have produced disappointing results, inducing weak Th1 responses and in some cases causing exacerbated disease (Wedlock, Vesosky et al. 2000; Wedlock, Keen et al. 2002). More recently it was reported that different formulations of CFP with CpG ODN induced strong IFN-γ responses and provided significant level of protection although not as effective as BCG (Wedlock, Denis et al. 2005). More promising results were obtained when CFP and CpG ODN were used to boost BCG vaccination as this gave a superior level of protection compared to BCG (Wedlock, Denis et al. 2005). These results show that both protein/adjuvant preparations and DNA vaccines can be used in combination with BCG to provide an enhanced level of protection compared to BCG alone.

1.4.1.4 Attenuated viral vectors

To date three main viral vectors have shown promise in prime-boost strategies, these are modified vaccinia Ankara (MVA), fowlpox and adenovirus. These live vaccines are

thought to be more effective at inducing strong Th1 type immune responses and long-lasting memory immune responses.

The immunogenicity of MVA and fowlpox virus expressing the mycobacterial Ag85A was investigated in cattle. Different combinations were compared and the strongest Th1 responses were observed in the animals that had received MVA-Ag85 and BCG. This regime resulted in a significantly higher frequency of Ag85-specifc IFN-γ producing cells compared to BCG alone, however the protective efficacy of this vaccination strategy has yet to be investigated (Vordermeier, Rhodes et al. 2004). The immunogenecity of MVA-Ag85/BCG in cattle echos that observed in humans and mouse models (McShane, Behboudi et al. 2002; McShane, Pathan et al. 2004).

Vaccination with replication-deficient recombinant adenovirus expressing Ag85A (Ad85A) has been reported to protect mice against *M. tuberculosis* infection (Wang, Thorson et al. 2004). In cattle, vaccination with BCG/Ad85A induced a strong Th1-type response measured by a cultured ELISPOT suggesting this heterologous prime-boost regime generates a memory response (Vordermeier, Huygen et al. 2006).

Taracha and co-workers, investigated the immunogenicity of administering different combinations of DNA, MVA and fowlpox expressing Ag85A in cattle. Antigen-specific IFN-γ responses were induced in animals that were primed with either DNA or fowlpox expressing Ag85A and boosted with MVA-Ag85 (Taracha, Bishop et al. 2003). It is not clear how these responses compared with those induced by BCG but these results provide promising data on vaccination strategies that may not require the implementation of new diagnostic test.

Other avenues of research into developing more effective TB vaccines for cattle include: modified strains of BCG containing genes encoding immunogenic mycobacterial proteins and attenuated mutants of *M. bovis* (Horwitz, Harth et al. 2000; Horwitz, Harth et al. 2006; Buddle, Skinner et al. 2002) . To date, the most promising results have been achieved by heterologous prime-boost vaccination strategies involving BCG and a subunit vaccine or a recombinant virus. One of the advantages of developing new TB vaccines for cattle is that a potential vaccine can be rigorously tested in experimental challenge trials.

1.4.2 Vaccines against Human Tuberculosis

In 1993, the WHO declared TB a global emergency, this lead to the resurgence in efforts to develop TB vaccines that are more effective than the current BCG. At present BCG is administered at birth and forms part of the WHO expanded Programme on Immunisation. When given at birth BCG provides a significant degree of protection against tuberculous meningitis and military TB during childhood (Rodrigues, Diwan et al. 1993). In contrast, BCG does not consistently protect against adult pulmonary TB and has been shown in many clinical trials and observational studies to induce a level of protection ranging from 0%-80% (Fine 1989; Fine, Carneiro et al. 1999). A number of explanations have been proposed for the variable efficacy of BCG including pre-sensitisation, host genetics, nutritional status, age and strain of BCG used. A body of evidence exists in support of the reduced efficacy of BCG in sensitised individuals. Exposure to environmental mycobacteria can result in the development of an anti-mycobacterial immune response which has been proposed to interfere with the ability of BCG to induce an immune response. Similar problems are encountered in cattle that have been sensitised by exposure to environmental mycobacteria. Thus, cattle may provide a useful model in which to determine the ability of potential vaccines to induce protective immune responses in sensitised individuals.

The design of a thereapeutic vaccine that could be used in individuals already infected with *M. tuberculosis* or individuals co-infected with HIV, should take into account the findings of Robert Koch. In 1891, Robert Koch reported that intradermal challenge with whole organisms or culture filtrate when administered to *M. tuberculosis* infected guinea pigs resulted in necrosis and healing at the injection site with similar lesions occurring in the original tuberculous lesion (Koch 1891). In humans with skin TB, Koch found that subcutaneous injection of larger amounts of culture filtrate induced necrosis in established tuberculous lesions and lead to the cure of skin TB. However, when humans with TB that involved the lungs or spine were injected with culture filtrate, the results were disastrous as this greatly exacerbated the disease (Koch 1891). Thus, the Koch reaction describes the development of immunopathology by administration of mycobacteria or mycobacterial products to *M. tuberculosis*-infected individuals. On this basis a prospective therapeutic vaccine should not be too immunogenic to avoid the induction of such a reaction. Encouragingly, the subunit

vaccine MVA-Ag85 does not appear to induce a Koch reaction in individuals already sensitised by exposure to environmental mycobacteria (McShane, Pathan et al. 2004; McShane, Pathan et al. 2005; Ibanga, Brookes et al. 2006).

Two main avenues of TB vaccine development are currently being investigated: one aims to replace BCG with a recombinant strain of BCG or with an attenuated strain of *M. tuberculosis*. The other involves boosting the current neonatal BCG vaccine with a subunit vaccine expressing immunodominant mycobacterial antigens. Based on these strategies several vaccine candidates have entered phase I or II clinical trials. These include a heterologous prime-boost strategy combining priming with BCG and boosting with MVA-Ag85A (Goonetilleke, McShane et al. 2003; McShane, Pathan et al. 2004). Two protein based subunit vaccines, the first involves the administration of a recombinant fusion protein immersed in AS02 adjuvant (Mtb72f) (Skeiky, Alderson et al. 2004) and the other is a recombinant fusion protein comprised of mycobacterial antigens ESAT-6 and Ag85B (Olsen, Williams et al. 2004; Langermans, Doherty et al. 2005). Two recombinant BCG strains are also currently being trialled in humans, these are rBCG30 which expresses large amounts of the 30 kDa mycobacterial antigen 85B (Horwitz, Harth et al. 2000) and a rBCG transfected with the membrane perforating listeriolysin of Listeria monocytes (Grode, Seiler et al. 2005).

In conclusion, a prospective vaccine should be effective in the face of prior sensitisation by BCG vaccination and/or exposure to environmental mycobacteria. Moreover the vaccine should be safe and effective in people with latent TB or co-infected with HIV. With the success of the current ongoing vaccination trials it is likely that the next few years may be a productive time for TB vaccine development.

1.5 Diagnostic techniques for the detection of Bovine TB

The tuberculin skin test measures the development of a delayed type hypersensitivity induced by the injection of purified protein derivative from *M. bovis* culture (PPD-B). In some countries the comparative skin test is utilised in which PPD-B and PPD from the environmental mycobacteria *M. avium* (PPD-A) are injected simultaneously at different sites. The difference in the DTH reaction as measured in skin thickness

indicates whether the animal is infected with *M. bovis* or is displaying a non-specific DTH reaction because of sensitisation by environmental mycobacteria. The comparative skin test is thought to be more effective as there is a high degree of conservation and cross-reactivity between the *M. bovis* and *M. avium*. The sensitivity of the skin test has been reported to be between 70-75% (Cousins 2001).

Problems with the skin test are numerous and include the lack of discrimination between *M. bovis* infected animals and animals sensitised by exposure to *M. avium*. It is possible that infected animals may respond equally well to both PPD-A and PPD-B and therefore no difference would be observed in the DTH reaction and these animal would escape detection. Furthermore not all infected animals will respond highly in the DTH reaction as the T-helper 1 (Th1) type immune response of animals with severe disease may be suppressed. One limitation of the tuberculin skin test is that it can not be used to re-test animals within 60 days of the previous test as it induces the suppression of immune responses to mycobacterial antigens (Lepper, Pearson et al. 1977; Radunz and Lepper 1985; Thom, Morgan et al. 2004) The mechanisms of the immunosuppressive effect of intradermal injection of PPD are unclear but involve a down-regulation of T cell proliferation and the induction of an antibody response to mycobacterial antigens (Doherty, Bassett et al. 1996; Lyashchenko, Whelan et al. 2004).

It is likely that a vaccine against bovine TB will interfere with the current tuberculin skin test which will not discriminate between vaccinated and vaccinated/diseased animals. This is one of the reasons why BCG vaccination has not been approved for use in cattle.

Attempts to improve on the methods of detection of *M. bovis* infected animals produced a test based upon the production of IFN-γ by PPD-B or PPD-A stimulated whole blood. This *in vitro* IFN-γ test showed an increased sensitivity and specificity compared to the tuberculin skin test (Wood and Rothel 1994; Whipple, Palmer et al. 2001). This test is now approved under European Union directives for use in the re-testing of animals that are negative in the tuberculin skin test or animals giving a positive skin test that have been potentially sensitised by environmental mycobacteria. However, this IFN-γ test still relies on responses to PPD-B which contains broad range

of antigens that are likely to be expressed by vaccination and infection. A more discriminatory test would measure responses to antigens that are only expressed by infection with *M. bovis* and are absent from environmental mycobacteria and potential vaccine candidates such as BCG. Efforts have focused on the use of CFP-10 and ESAT-6, mycobacterial proteins encoded by the RD1 region of *M. bovis* which is deleted in all strains of BCG (Mahairas, Sabo et al. 1996; Behr, Wilson et al. 1999; Garnier, Eiglmeier et al. 2003). Strong T cell responses are commonly detected to both ESAT-6 and CFP-10 in *M. bovis* infected animals but not in BCG-vaccinated animals (Buddle, Parlane et al. 1999; Buddle 2001; Vordermeier, Whelan et al. 2001; Buddle, McCarthy et al. 2003; Buddle, Wedlock et al. 2003). In humans, these two antigens have been shown to contain both $CD4^+$ and $CD8^+$ T cell epitopes. In BCG-vaccinated cattle subsequently infected with *M. bovis*, immune responses to ESAT-6 and CFP-10 were only observed in animals that developed disease while protected animals showed weaker responses to these antigens (Vordermeier, Chambers et al. 2002). Antigens encoded by RD regions 1, 2 and 14 expressed by *M. bovis* but absent in BCG have also been used in combination with ESAT-6 and CFP-10 which may help to discriminate between different stages of *M. bovis* infection (Cockle, Gordon et al. 2002; Mustafa 2002).

1.6 Innate Immune Responses in Tuberculosis

The innate immune system provides the first line of defence against pathogens. Innate immune cells which mediate this rapid non-specific response are activated by the binding of pattern recognition receptors (PRRs) with pathogen associated molecular patterns (PAMPS). PAMPS are conserved molecular structures expressed by a variety of pathogens and include mannans, lipopolysaccharides, formylated peptides and lipotechoic acids (Medzhitov and Janeway 2000). Innate responses are either humoral, which include the complement system, or cellular, such as natural killer cells (NK cells) and phagocytes (e.g.macrophages, granulocytes and dendritic cells).

PRR receptors include the Toll-like receptors (TLR), mannose receptor (MR), DEC-205, complement receptor, scavenger receptors and integrins (Aderem and Underhill 1999).

TLRs are essential for the recognition of microbes and initiation of immune responses. The toll protein was originally identified in Drosophila and subsequently homologues were discovered in mammals (Medzhitov, Preston-Hurlburt et al. 1997). In mammals, the cytoplasmic domain of TLR is homologous to the signalling domain of IL-1 receptor. Activation of TLR through ligand binding induces dimerisation and interactions with signalling molecules. TLR signalling can be initiated via the adaptor molecule myeloid differentiation primary-response protein 88 (Myd88) or through a MyD88-independent fashion. Downstream signalling events involve kinases IRAK (IL-1R-associated kinase), transforming growth factor-β (TGF-β)-activated kinase (TAK1), TAK1-binding protein (TAB1), TAB2 and tumour necrosis factor (TNF)-receptor associated factor 6 (TRAF6). These molecules initiate translocation of transcription factors such as NF-κB to the nucleus leading to the expression of a number of immune response genes such as those encoding pro and anti-inflammatory cytokines (Krutzik, Tan et al. 2005). TLR signalling can also initiate differentiation of monocytes into Mϕ and dendritic cells, and in Mϕ upregulates phagocytosis of bacteria and apoptotic cells (Blander and Medzhitov 2004; Doyle, O'Connell et al. 2004; Shiratsuchi, Watanabe et al. 2004).

To date, at least 10 functional TLRs have been identified in humans, these differ in cellular distribution, localisation and respective ligands (Akira 2004). In cattle sequences are available for TLRs 1-10 and the expression of these varies in cells from different origins (Werling, Piercy et al. 2006). Alveolar Mϕ and bone-marrow DCs expressed the greatest diversity of TLRs, whilst expression of these on monocyte-derived Mϕ or DC was more variable.

1.6.1 Recognition and Uptake of Mycobacteria

The initial interaction between mycobacteria and the host is one of the key factors that can influence the disease outcome: clearance, progressive acute infection or containment (Fenton and Vermeulen 1996; Flynn and Chan 2001). Although *M. tuberculosis* can infect any tissue, the airways represent the most common port of entry for *M. tuberculosis* and the primary site of infection. One of the first cells to come into contact within inhaled mycobacteria is the alveolar Mϕ. The alveolar Mϕ is the main cellular host of mycobacteria. In addition DCs are also present in the respiratory tract

and similar to alveolar Mϕ, harbour and phagocytose mycobacteria. Mycobacteria and mycobacterial products can bind a number of different receptors, some of which mediate the phagocytosis of the mycobacteria and others activate anti-microbial functions of the phagocytic cell.

Phagocytosis of mycobacteria can occur through the binding of opsonised or non-opsonised *M. tuberculosis* to complement receptors (CR) 1, 3 and 4, mannose receptor, surfactant protein A (Sp-A), type A scavenger receptor and Fcγ receptor (Armstrong and Hart 1975; Zimmerli, Edwards et al. 1996; Zimmerli, Majeed et al. 1996). Opsonisation of mycobacteria by complement factor C3 enhances the binding and uptake of *M. tuberculosis* by the complement receptors (Schlesinger 1993; Hirsch, Ellner et al. 1994; Aderem and Underhill 1999). In addition, non-opsonised pathogenic mycoabcteria can also bind directly to CR3 and CR4 whereas non-pathogenic mycobacteria cannot (Cywes, Hoppe et al. 1997; Zaffran and Ellner 1997). The mannose receptor (MR) is one of the best characterised receptors for non-opsonised *M. tuberculosis* that binds terminal mannose residues on the mycobacteria (Schlesinger 1996; Schlesinger 1996).

Opsonisation can enhance the binding of *M. tuberculosis* to epithelial cells and alveolar Mϕ, in addition to complement components, this can occur through binding of surfactant A protein, and mannose-binding lectin (MBL) (Downing, Pasula et al. 1995; Gaynor, McCormack et al. 1995; Garred, Madsen et al. 1997).

Internalisation of mycobacteria through binding of CR or MR is advantageous to the mycobacteria as these pathways do not initiate the oxidative burst process, thereby the mycobacteria are not exposed to reactive oxygen species (Aderem and Underhill 1999; Astarie-Dequeker, N'Diaye et al. 1999; Astarie-Dequeker, N'Diaye et al. 1999). Similarly phagocytosis of Sp-A opsonised *M. tuberculosis* by alveolar Mϕ suppresses reactive nitrogen intermediates (Pasula, Wright et al. 1999).

M. tuberculosis has been reported to infect alveolar epithelial cells and may bind through interactions of fibronectin on the epithelial cells and mycobacterial Ag85 complex which is a member of the fibronectin-binding protein family (Wiker and Harboe 1992; Bermudez and Goodman 1996).

Thus, the phagocytosis of mycobacteria can occur via a number of mechanisms, involving a number of host cell receptors. The internalisation of *M. tuberculosis* by the

different receptors may lead to differential effects on the survival of *M. tuberculosis*, signal transduction and immune activation.

Recognition of mycobacteria and mycobacterial products is a crucial step in the initiation of cytokine production and anti-microbial effector functions of Mϕ and is mediated by a number of PRRs including TLRs. Mycobacteria can trigger different TLRs through the binding of different mycobacterial components. Interactions of mycobacteria with TLRs expressed by Mϕ and DC may have important implications for development of the appropriate adaptive immune response.
Recognition of the mycobacterial cell wall component lipoarabinomannam (LAM) appears to be similar to that of gram-negative bacterial lipopolysaccharide (LPS). Plasma LPS-binding protein associates with LAM and transferrs it to the CD14 surface receptor which generally co-localises with TLR4 (Fenton MJ 1998). However, TLR2 but not TLR4 is essential for the signalling of mycobacterial LAM (Means, Lien et al. 1999; Underhill, Ozinsky et al. 1999). TLR4 has been reported to bind an undefined heat-liable cell associated mycobacterial factor (Means, Wang et al. 1999).

The 19kDa lipoprotein of *M. tuberculosis* was reported to stimulate the production of IL-12 and reactive nitrogen intermediates via interactions with TLR2 on Mϕ (Brightbill, Libraty et al. 1999). Subsequent studies have indicated that stimulation of TLR2 on cells by 19 kDa lipoprotein of *M. tuberculosis* also initiates the production of IL-10 which is completely abrogated by inhibition of TLR2 (Drennan, Nicolle et al. 2004; Jang, Uematsu et al. 2004) . In support of this inhibitory role, the 19 kDa lipoprotein inhibits MHC class II presentation and responsiveness to IFN-γ in Mϕ in a TLR-2 dependent manner (Noss, Pai et al. 2001; Fortune, Solache et al. 2004). The suppressive effects of this *M. tuberculosis* antigen may represent a key survival strategy for mycobacteria.

Mice deficient in TLR2, TLR6 or TLR2 and TLR4 are no more susceptible to low dose *M. tuberculosis* infection than wild-type (wt) mice (Reiling, Holscher et al. 2002; Sugawara, Yamada et al. 2003). In contrast, mice lacking TLR2 are more susceptible to high dose *M. tuberculosis* infection and mice deficient in MyD88 are highly susceptible to low and high dose *M. tuberculosis* infection compared to wt mice (Reiling, Holscher

et al. 2002). In the MyD88 knockout (KO) mice expression of IFN-γ, TNF-α, IL-12 and iNOS was reduced but not abolished (Fremond, Yeremeev et al. 2004; Scanga, Bafica et al. 2004). Furthermore T cells from these mice were responsive to *ex vivo* stimulation and BCG-vaccination prior to *M. tuberculosis* infection conferred a low level of protection. These findings indicate that MyD88 independent mechanisms may lead to the production of pro-inflammatory cytokines and anti-microbial effector proteins by *M. tuberculosis* infected Mϕ in the MyD88 KO mice.

In addition to TLR2 and TLR4, TLR9 binds unmethlyated CpG motifs present in bacterial DNA and therefore may also be involved in immune recognition of mycobacteria (Hemmi, Takeuchi et al. 2000). More recently a role for TLR9 in the induction of IL-12 production by Mϕ and DC in response *M. tuberculosis* infection was demonstrated as blocking of TLR9 significantly reduced IL-12 secretion. In addition this decrease in IL-12 was observed in *M. tuberculosis* infected TLR9 KO mice and was accompanied by a decrease in the percentage of IFN-γ producing *M. tuberculosis* reactive $CD4^+$ T cells. Although mice lacking either TLR2 or TLR9 are not any more susceptible to infection with *M. tuberculosis* than wt mice, mice deficient in both TLR9 and TLR2 display a significantly enhanced susceptibility (Bafica, Scanga et al. 2005).

Interactions of mycobacteria with multiple TLRs is likely to be important to the control of infection and the generation of effective T helper 1 responses. It is possible that some redundancy exists among the different TLR receptors and signalling through these receptors during *M. tuberculosis* infection is likely to occur through MyD88 dependent and independent mechanisms. Furthermore cross-talk between TLRs and receptors involved in phagocytosis adds a further dimension to the role of these receptors in the control of *M. tuberculosis* infection.

1.6.2 Interactions with Macrophages

Macrophages (Mϕ) internalise and phagocytose a broad range of particles from harmless cell debris to infectious pathogens. The receptors utilised by the uptake of dangerous pathogens can lead to the activation of a range of anti-microbial mechanisms of the Mϕ. Activation of mycobactericidal functions of Mϕ can be further enhanced by

IFN-γ which can act alone or in synergy with TNF-α and through vitamin D receptor binding. Pro-inflammatory cytokines IFN-γ and TNF-α induce the production of nitric oxide (NO) and reactive nitrogen intermediates (RNI) by Mϕ through the action of inducible form of nitric oxide synthase (iNOS2) (Ding, Nathan et al. 1988). In cattle activation of Mϕ with IFN-γ prior to *M. bovis* infection, increased the ability of Mϕ to inhibit viability of the mycobacteria (Aldwell, Wedlock et al. 2001).

In support for the involvement of vitamin D, specific polymorphisms in vitamin D receptor have been associated with either increased susceptibility or resistence to *M. tuberculosis* infection (Bellamy, Ruwende et al. 1999). In addition the active metabolite of vitamin D enhances the ability of Mϕ to suppress *M. tuberculosis* growth (Rockett, Brookes et al. 1998); (Rook, Steele et al. 1986).

Mϕ are potent antimicrobial components of the innate immune system. Mϕ can mediate the killing of microbes via a number of mechanisms which include phago-lysosomal fusion, generation of ROI by the oxidative burst and the production of RNI via the NOS2-dependent cytotoxic pathways. ROI and RNI are delivered to through the endosomal system to phagosomes containing mycobacteria and directly damage the mycobacterial DNA.

The formation of phagolysosomes occurs as part of the dynamic process initiated by the phagocytosis of microbes. Upon phago-lysosomal fusion, degradation of microbes is mediated by lysosomal hydrolases and acidification of the phagolysosome. However, it is known that *M. tuberculosis* inhibits maturation of the phagosome and thereby inhibits the fusion with lysosomes.

NO and RNI are generated from L-arginine by nitric oxide synthase type 2 (NOS2). Production of these chemically reactive micromolecules is induced by activation of Mϕ and is highly toxic to invading microbes. RNI in a liquid and gaseous form can kill *M. tuberculosis* (Long, Light et al. 1999). Certain forms of RNI are more effective at killing different mycobacteria for example *M. tuberculosis* is senisitive to NO but more resistant to peroxynitrite than other mycobacteria (Yu, Mitchell et al. 1999). The essential role of RNI production during *M. tuberculosis* infection was demonstrated by

the inhibition of NOS2 activity in infected Mϕ and in *M. tuberculosis* infected mice which lead to exacerbated infection (Denis 1991; Flesch and Kaufmann 1991; Chan, Xing et al. 1992; Chan, Tanaka et al. 1995; MacMicking, Xie et al. 1997; MacMicking, North et al. 1997). Moreover NOS2 deficient mice succumb prematurely to infection with *M. tuberculosis* due uncontrolled growth of the mycobacteria (MacMicking, Xie et al. 1997). In humans, NOS2 was reported to be expressed by Mϕ present in the lungs of TB patients (Nicholson, Bonecini-Almeida Mda et al. 1996). In some studies the expression of NOS2 failed to correlate with mycobactericidal activity, in these circumstances it is possible that the activity of NOS2 was limited due to absence of a synergising pathway (Rich, Torres et al. 1997). Another possibility is that the mycobacteria are inhibiting NOS2 activity and are resistant to the antimicrobial effects of RNI. In cattle, expression of NO was up-regulated in bovine Mϕ in response to infection with either *M. bovis* or BCG (Aldwell, Wedlock et al. 1996).

The role of oxidative burst and generation of ROI by activated Mϕ in the control of *M. tuberculosis* infection is controversial. Hydrogen peroxide was one of the first effector molecules to be identified as mediating anti-mycobacterial effects of Mϕ (Walker and Lowrie 1981). However the ability of ROI to kill *M. tuberculosis in vitro* remains to be established as mycobacterial components such as LAM and phenoic glycolipid I (PGL-I) bind free oxygen radicals (Chan, Fujiwara et al. 1989). However, mice deficient in functional NADPH-oxidase required for the production of superoxide are unable to control mycobacterial replication in the early stages of infection (Cooper, Pearl et al. 2000; Cooper, Segal et al. 2000). The difference in the requirement for ROI in the immuninity to *M. tuberculosis* infection of humans and mice may reflect the fact that mice are not the natural host of *M. tuberculosis* infection.

Apoptosis has been proposed as an additional effector mechanism by which Mϕ can inhibit mycobacterial growth as *in vitro* experiments showed that apoptosis of infected Mϕ but not necrosis resulted in reduced numbers of mycobacteria (Molloy, Laochumroonvorapong et al. 1994; Keane, Balcewicz-Sablinska et al. 1997; Placido, Mancino et al. 1997). In response to *M. tuberculosis* infection, Mϕ express high levels of TNF-α which may act to induce apoptosis of infected Mϕ as binding of some TNF-receptors have been shown to induce apoptosis. Supprisingly more virulent

mycobacteria induce less host cell apoptosis compared to attenuated strains (Keane, Balcewicz-Sablinska et al. 1997). This was attributed to the induction and release of soluble TNF-α receptor by virulent *M. tuberculosis* (Balcewicz-Sablinska, Keane et al. 1998).

However, the induction of apoptosis in infected Mϕ has been reported to either reduce mycobacterial numbers or have no effect on the viability of the mycobacteria. This difference may be related to the apoptotic pathway that is utilised as a number of mechanisms can lead to the induction of apoptosis which include the actions of granzymes present in cytotoxic granules, ligation of FAS, binding of ATP and binding of TNF-α to specific TNFR death receptors. In addition to these the mycobacteria inside the Mϕ may also directly induce apoptosis of the host cell.

The natural-resistance-associated macrophage protein (Nramp1) was proposed to be involved in activation of macrophages and killing of mycobacteria. Nramp1 becomes part of the phagosomal membrane and is involved in the transport of metal ions. In humans polymorphisms in the promoter region of Nramp1 resulted in a decreased gene expression and was associated with an increased susceptibility to *M. tuberculosis* infection (Bellamy, Ruwende et al. 1998; Cervino, Lakiss et al. 2000). In mice, Nramp 1 was found to be important to the maturation and acidification of the phagosome (Hackam, Rotstein et al. 1998).

1.6.3 Interactions with DC

DCs are potent inducers of T cell responses as their primary function is antigen presentation. Activation of immature DCs can occur in response to infection with mycobacteria resulting in the migration of the immature DCs from the lung to the draining lymph nodes. This migration process is associated with maturation of the immature DC and leads to an up-regulation of molecules involved in the interaction with and stimulation of naïve T cells in the lymph nodes such as MHC class II molecules, CD80, CD86 and CD40. Furthermore mature DCs have the capacity to produce cytokines such as IL-12 which promote the differentiation of Th1 responses.

The intracellular behaviour of *M. tuberculosis* in DC, is different to that in Mϕ, as *M. tuberculosis* is unable to replicate in DCs. This may be attributed to the fact *M. tuberculosis* is contained within DCs disconnected from the host-cell recycling pathway and does not have access to exogenous nutrients unlike inside Mϕ (Tailleux, Neyrolles et al. 2003). This differential behaviour may reflect differences in the receptors involved in uptake of mycobacteria by DC and Mϕ. Although human DCs express CR3 and MR, binding of mycobacteria to DCs is predominantly mediated by DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). It was reported that blocking of DC-SIGN reduced mycobacterial binding to DC by up to 90%, whereas binding was not significantly affected by blocking of CR3 and MR (Tailleux, Schwartz et al. 2003). The ligand for DC-SIGN was found to be ManLAM the polymannosylated lipoarabinomanan a component of the cell wall of slow-growing mycobacteria such as *M. tuberculosis* and *M. bovis*.

DC-SIGN plays an important role in the uptake of mycobacteria by DC whereas its expression on Mϕ is restricted. A recent report showed that DC-SIGN expression is up-regulated in Mϕ derived from bronchoalveolar lavage (BAL) of *M. tuberculosis* infected individuals (Tailleux, Pham-Thi et al. 2005). Thus the role of this receptor in the uptake of *M. tuberculosis* by Mϕ requires further clarification.

These reports suggest that the function of DCs during infection with mycobacteria may be twofold. Infected DCs harbour *M. tuberculosis* within an environment that constrains *M. tuberculosis* replication and these cells then migrate to the LN and stimulate *M. tuberculosis* specific T cells. However, the migration of DCs containing live *M. tuberculosis* to the LN may be detrimental to the host as it provides a means by which *M. tuberculosis* can spread to other tissues.

1.6.4 Involvement of Cytokines

As mentioned, recognition of mycobacteria by phagocytic cells leads to the activation of the cell and the production of cytokines. These cytokines can exert either a paracrine or autocrine effect. The cytokine network induced by mycobacterial infection involves the actions of both pro- and anti-inflammatory cytokines that may play a crucial role in the outcome of the infection.

The network of proinflammatory cytokines induced during *M. tuberculosis* infection include TNF-α, IL-12, IL-1β, IL-18, IL-15 and IFN-γ. In addition, IL-6 has both a pro and anti-inflammatory properties. TNF-α and IL-1β are produced mainly by monocytes, macrophages and DCs in response to mycobacteria or mycobacterial products (Valone, Rich et al. 1988; Roach, Barton et al. 1993; Henderson, Watkins et al. 1997). Both of these cytokines play crucial roles in the formation of the granuloma and the control of *M. tuberculosis* replication as mice deficient in either TNF-α or IL-1β are unable to control *M. tuberculosis* growth, form defective granulomas and display an increased susceptibility to *M. tuberculosis* infection (Bean, Roach et al. 1999; Juffermans, Florquin et al. 2000); (Flynn, Goldstein et al. 1995). In humans both of these cytokines have been identified at the site of infection (Law, Weiden et al. 1996). In addition *M. tuberculosis* specific $CD8^+$ and $CD4^+$ T cells also produce TNF-α and this cytokine may be an important mediator of apoptosis of infected Mϕ leading to a reduction in *M. tuberculosis* viability. In cattle, *M. bovis* and BCG infected Mϕ upregulate mRNA expression of TNF-α, IL-6 and IL-1β (Aldwell, Wedlock et al. 1996; Wedlock, Aldwell et al. 1999).

Similar to TNF-α and IL-1β, IL-12 is produced mainly by phagocytic cells in response to *M. tuberculosis* (Fulton, Johnsen et al. 1996; Ladel, Szalay et al. 1997). IL-12 biases the generation of IFN-γ producing T cells and provides an important link between the innate and adaptive immune responses against infection with mycobacteria (Cooper, Magram et al. 1997; Okamura, Kashiwamura et al. 1998). Increased levels of IL-12 have been reported at the site of infection in TB patients (Bergeron, Bonay et al. 1997; Casarini, Ameglio et al. 1999). Furthermore humans carrying genetic mutations in the IL-12R or IL-12 p40 subunit display a reduced capacity to produce IFN-γ and an increased susceptibility to mycobacterial infections (Altare, Durandy et al. 1998; Altare, Lammas et al. 1998).

IL-18 has been proposed to act in synergy with IL-12 to induce IFN-γ production (Okamura, Kashiwamura et al. 1998). A protective role for IL-18 in the control of mycobacterial infections was demonstrated in IL-18 KO mice as these mice are highly susceptible to *M. tuberculosis* and BCG infection (Sugawara, Yamada et al. 1999).

Furthermore production of IL-18 was reduced in patients with active TB who also display a reduced production of IFN-γ (Vankayalapati, Wizel et al. 2000; Vankayalapati, Wizel et al. 2001). A number of other pro-inflammatory cytokines are likely to be involved in immunity to mycobacteria such as IL-15 which is important in induction and maintanence of memory T cell responses (Berard, Brandt et al. 2003). In addition, IL-23 and IL-17 have been proposed to be involved in the proliferation and activation of Th1 cells and therefore may be important to immunity to mycobacteria (Khader SA et al 2005).

IL-6 is a pleiotropic cytokine, produced by a variety of cells including Mϕ, T cells, endothelial cells and fibroblasts (Flesch and Kaufmann 1993; Law, Weiden et al. 1996; Jang, Uematsu et al. 2004). During the early stages of *M. tuberculosis* infection IL-6 is produced by infected Mϕ (Flesch and Kaufmann 1993; Hoheisel, Izbicki et al. 1998). In its pro-inflammatory capacity IL-6 induces the production of acute phase proteins and may influence the production of IFN-γ by innate immune cells early in the infection process with *M. tuberculosis*. IL-6 deficient mice displayed an increased susceptibility to *M. tuberculosis* infection which appeared to be related to a decrease in IFN-γ production by innate immune cells (Ladel, Blum et al. 1997; Saunders, Frank et al. 2000). In contrast production of IL-6 by *M. tuberculosis* infected Mϕ inhibited the response of both the infected Mϕ and adjacent uninfected Mϕ to IFN-γ (Nagabhushanam, Solache et al. 2003). Furthermore IL-6 has been shown to inhibit the production of TNF-α and IL-1β by Mϕ (Schindler, Mancilla et al. 1990).

The production of anti-inflammatory cytokines such as IL-10 and TGF-β can inhibit the protective effects of the pro-inflammatory cytokines and can also inhibit other effectors mechanisms of Mϕ and mycobacterial specific T cells. IL-10 has been shown to be produced by *M. tuberculosis* and *M. bovis* infected Mϕ. and down-regulates the production of TNF-α, IL-10 and IFN-γ (Flesch, Hess et al. 1994; Fulton, Cross et al. 1998; Hirsch, Toossi et al. 1999). TGF-β is produced by monocytes and DCs in response to mycobacterial products. In particular LAM induces the production of both IL-10 and TGF-β and these cytokines are produced in excess during active TB at the site of infection (Toossi, Gogate et al. 1995; Dahl, Shiratsuchi et al. 1996; Condos, Rom et al. 1998). TGF-β suppresses Mϕ activation and antigen presentation, and

inhibits cell proliferation and IFN-γ production (Dahl, Shiratsuchi et al. 1996; Toossi and Ellner 1998).

1.6.5 Involvement of chemokines

Chemokines function to recruit inflammatory cells to the site of infection and a number of chemokines have been implicated to be involved in cell trafficking in TB. These include the production of IL-8, monocyte chemoattractant protein 1 (MCP-1) and RANTES. IL-8 is produced by phagocytic cells upon the uptake of *M. tuberculosis* and in response to recognition of LAM (Zhang, Broser et al. 1995). In TB patients, IL-8 was found to be expressed in BAL, LN and plasma (Bergeron, Bonay et al. 1997; Kurashima, Mukaida et al. 1997; Juffermans, Verbon et al. 1999). Increased levels of IL-8 were found in patients who died from TB (Friedland, Hartley et al. 1995). MCP-1, which is produced by and acts on macrophages and monocytes (Kasahara, Tobe et al. 1994). Mice deficient in MCP-1 fail to form granulomas in response to infection with *M. tuberculosis*. In addition mice deficient in the C-C chemokine receptor 2 (CCR2), to which MCP-1 binds, show reduced granuloma formation and succumb quickly to *M. tuberculosis* infection (Lu, Rutledge et al. 1998). Elevated levels of MCP-1 and RANTES were found in patients with active TB (Kurashima, Mukaida et al. 1997).

In summary, the innate immune response begins with the initial uptake of mycobacteria by alveolar Mϕ. This interaction results in activation of the Mϕ leading to the production of cytokines, chemokines and mycobactericidal effector molecules which influences the ensuing adaptive response. The ability of an individual to recognise and respond to mycobacteria may be genetically determined as a number of functional gene polymorphisms which affect macrophage function have been associated with susceptibility to TB (Bellamy, Ruwende et al. 1998; Bellamy, Ruwende et al. 1999; Wilkinson, Patel et al. 1999).

1.6.6 Contribution of Natural killer (NK) cells

NK cells are large granular lymphocytes of the innate immune system that are conserved among mammals, birds and fish (Gobel, Chen et al. 1994; Tekin and Hansen

2002) In humans, NK cells comprise up to 15% of peripheral blood lymphocytes and form an important link between the innate and adaptive immune responses. Their primary role is in the early detection and killing of virally infected cells or tumour cells, by targeting cells that express low or no MHC class I molecules. In addition to cytotoxicity, activated NK cells produce a number of cytokines that bias the development of T helper 1 type adaptive immune responses(Martin-Fontecha, Thomsen et al. 2004).

NK cells form part of the innate immune response induced by infection with *M. tuberculosis* and *M. bovis*, through the production of IFN-γ, lysis of infected Mϕ and killing of mycobacteria (Batoni, Esin et al. 2000; Brill, Li et al. 2001; Ferlazzo, Morandi et al. 2003; Endsley, Furrer et al. 2004) (Storset AK et al 2003). Thus NK cells are key effector cells that control mycobacterial replication and dissemination early in the infection process.

In *M. tuberculosis*-infected mice, NK cells are recruited into the lungs within the first seven days of infection and these cells produced large amounts of IFN-γ. However depletion of NK cells in *M. tuberculosis* infected mice does not lead to an increased susceptibility to *M. tuberculosis* infection whereas depletion of NK cells in *M. avium* infected mice results in an increased bacterial burden (Junqueira-Kipnis, Kipnis et al. 2003)

In humans, NK cells that express the NK cell-activating receptor p46 (NKp46) were found to lyse *M. tuberculosis*-infected monocytes. Expression of NKp46 was reduced in patients with active TB, suggesting a protective role for this subset of NK cells in controlling *M. tuberculosis* infection in humans (Vankayalapati, Wizel et al. 2002). Human NK cells can rapidly kill *M. tuberculosis*-infected Mϕ via a mechanism that requires cell contact and is independent of IFN-γ production, FAS-FASL interactions and exocytosis of granules (Brill, Li et al. 2001).

In cattle, expression of NKp46 defines a population of NK cells that constitute up to 5% of bovine PBMC depending on the age of the animal (Storset, Kulberg et al. 2004). Activation of $NKp46^{+}CD3^{-}$ cells with recombinant IL-12 and IL-15 increased the expression of perforin, granulysin and IFN-γ by these NK cells. Furthermore cytokine

activated NK cells reduce BCG numbers when cultured with BCG-infected Mϕ (Endsley, Endsley et al. 2006). Antigenic activation of NKp46$^+$ cells from both BCG-vaccinated and non-vaccinated animals induces proliferation, production of IFN-γ and inhibition of mycobacterial replication (Denis, Keen et al. 2006). In addition, NK cells may aid the development of Th1 responses during *M. bovis* infection by enhancing the production of IL-12 by infected Mϕ (Denis, Keen et al. 2006).

1.6.7 Involvement of Gamma-delta (γδ) T cells

γδ T cells were first identified in 1986 and their physiological role and respective ligands are not yet clearly defined (Brenner, McLean et al. 1986). In cattle and other ruminants, γδ T cells constitute a large proportion of the circulating lymphocyte population, and can represent up to 75 % of T cells in young cattle and up to 40% in adult animals (Mackay and Hein 1989; Hein and Mackay 1991) In contrast, between 1-10% of CD3$^+$ cells in human PBMC express the γδ TCR and 2-3% of murine PBMC express the γδ TCR (Bucy, Chen et al. 1989; Itohara, Nakanishi et al. 1989) γδ T cells recognise and respond to lipids and small non-peptide phosphate or amine containing antigens. Recognition of phosphoantigens by γδ T cells is TCR dependent but not restricted or dependent on any known MHC or MHC-like molecule. Human γδ T cells have been shown to respond to akylphosphate, alkylamine and aminobisphosphonate (Green, Lissina et al. 2004)

The involvement of γδ T cells in immune responses against *M. tuberculosis* infection of humans and mice is suggested by the accumulation of these cells at the site of infection (Modlin, Pirmez et al. 1989; Griffin, Harshan et al. 1991). However, in mice these cells do not form part of the protective immune response against BCG infection but were shown to be important for the control of early *M. tuberculosis* infection (Ladel, Blum et al. 1995; Ladel, Hess et al. 1995; Nabeshima, Hiromatsu et al. 1995)

In humans, the predominant population of *M. tuberculosis* reactive γδ T cells express a TCR encoded by Vγ9 and Vδ2 gene segments (Constant, Davodeau et al. 1994). A role for γδ T cells in human TB was further indicated by studies in patients with active pulmonary TB, which showed that the numbers of Vγ9Vδ2 *M. tuberculosis* reactive T cells present in blood and bronchoalveolar lavage (BAL) of these patients was reduced compared to healthy PPD-positive individuals (Li, Rossman et al. 1996).

In BCG-vaccinated individuals, this subset of γδ T cells was shown to proliferate in response to *M. tuberculosis* sonic extract but not after culture with live or UV-killed *M. tuberculosis* (Esin, Batoni et al. 1996). In addition to proliferation, *M.tb* reactive Vγ9Vδ2 T cells from PPD-positive individuals can kill *M. tuberculosis* infected Mϕ and reduced *M. tuberculosis* viability via the release of granule exocytosis. Subsequently, Vγ9Vδ2 T cells were found to mediate the killing of intracellular *M. tuberculosis* via the expression of perforin and granulysin whereas killing of extracellular *M. tuberculosis* was mediated solely by granulysin (Dieli, Troye-Blomberg et al. 2001).

In addition to their role in defense against *M. tuberculosis* infection, other subsets of γδ T cells may exert a suppressive role, as an increase in the ratio of Vγ1Vδ4 to Vγ9Vδ2 T cells was observed in the blood of anergic, skin-test negative TB patients, suggesting that Vδ1Vγ4 T cells may be inhibiting the DTH reaction (Baliko, Szereday et al. 1997; Szereday, Baliko et al. 2003) Moreover in patients co-infected with HIV and *M. tuberculosis* the number of circulating Vδ2 expressing T cells is reduced compared to healthy controls (Carvalho, Matteelli et al. 2002).
Thus, in human TB it is possible that different subsets of γδ T cells may exert different functions and that the Vγ9Vδ2 T cells play an important role in the protective immune response to *M. tuberculosis* infection.

In cattle two distinct subpopulations of γδ T cells have been identified based on upon expression of WC1, CD2 and CD8 as being $WC1^{+}CD2^{-}CD8^{-}$ and $WC1^{-}CD2^{+}CD8^{+}$. These two subpopulations are largely found in different tissues and have distinct TCR gene rearrangements (MacHugh, Mburu et al. 1997). WC1 belongs to the scavenger receptor cysteine rich domain family and exist as a number of isoforms (Wijngaard, Metzelaar et al. 1992). Although two human gene sequences have been identified which are more than 85% homologous to the bovine WC1 gene sequence, as yet there are no reports showing WC1 expression on human γδ T cells (Wijngaard, MacHugh et al. 1994). Likewise, it appears that WC1 is not expressed on murine γδ T cells. The function of WC1 has yet to be defined, it has been proposed to control the tissue-specific homing of γδ T cells (Wijngaard, MacHugh et al. 1994) and regulate IL-2 induced proliferation of γδ T cells (Takamatsu, Kirkham et al. 1997).

Activation of bovine $WC1^+$ γδ T cells by mycobacteria was demonstrated by an up-regulation of expression of CD25 and down-regulation of CD62L expression after in vitro culture with PPD-B or MBSE (Smyth, Welsh et al. 2001; Buddle, Wedlock et al. 2003; Waters, Rahner et al. 2003). These results indicate that γδ T cells are activated during *M. bovis* infection and activation may induce changes in the migratory capacity of these cells. Previous reports have shown that the percentage of $WC1^+$ T cells in blood decreases during early stages of *M. bovis* infection and increases in early lung lesions indicating that $WC1^+$ T cells are recruited to the site of infection (Pollock, Pollock et al. 1996; Cassidy, Bryson et al. 2001; Johnson, Gough et al. 2006).

The role of $WC1^+$ T cells in protective immune responses against *M. bovis* infection in cattle is unclear as a more recent study reported that a greater number of these $WC1^+$ T cells were present in the necrotic granulomas found in unprotected non-vaccinated animals compared to protected BCG-vaccinated animals (Johnson, Gough et al. 2006). Smith et al reconstituted severe combined immunodeficient mouse with bovine lymphoid tissue to generate a SCID-bo mouse. The role of $WC1^+$ γδ T cells in *M. bovis* infection was investigated and it was reported that antibody depletion of these cells in the SCID-bo mice resulted in an altered granuloma architecture. It is possible that $WC1^+$γδ T cells may be recruited to the site of infection during the early stages and function to attract other immune cells to the site of infection to form functional granuloma's (Smith, Kreeger et al. 1999).
The temporary depletion of $WC1^+$ T cells in cattle prior to infection with *M. bovis* did not significantly affect disease pathology. However, the proliferation of PPD-B stimulated PBMC from WC1-depleted animals was slightly lower than that of non-depleted animals. In addition, an increase in production of IL-4 was observed in the WC1-depleted animals (Kennedy, Welsh et al. 2002). These results suggest that $WC1^+$ T cells may be involved in the elicitation of Th1 T cells in response to *M. bovis* infection.

Mycobacteria-reactive γδ T cells from cattle produce IFN-γ in response to mycolylarabinogalactan peptidoglycan, a cell wall component of *M. bovis* (Vesosky, Turner et al. 2004). A report by Rhodes et al also demonstrated that purified γδ T cells

from *M. bovis* infected animals proliferated in response to a number of other mycobacterial antigens such as Ag85, ESAT-6, MPB83, MPB70, MPB64, heat-shock proteins 70 and hsp16.1. The proliferative responses of the γδ T cells to these antigens were higher in *M. bovis* infected animals than in non-infected animals. Furthermore a greater percentage of $WC1^+$ T cells from *M. bovis* infected animals proliferated in response MBSE compared to non-infected animals (Smyth, Welsh et al. 2001)
This indicates that γδ T cells from *M. bovis* infected animals are more responsive to mycobacteria than those from uninfected animals and is in agreement with Hoft et al who proposed that a type of γδ T cell memory exists in BCG-vaccinated human beings (Hoft, Brown et al. 1998).

In addition to antigen specific proliferation, the *M. bovis*-reactive γδ T cells enhanced the levels of IFN-γ and TGF-β in the cell cultures. Depletion of γδ T cells from PBMC stimulated with mycobacterial antigens enhances the proliferation of the remaining cells in the PBMC (Rhodes, Hewinson et al. 2001).

Hence a dual role for γδ T cells has been proposed involving the early production of IFN-γ and recruitment of T cells to the site of infection. The other function of *M. bovis* reactive γδ T cells is an inhibitory role in which these cells induce TGF-β production which may act to dampen down the immune response and restrict the amount of immunopathology induced by the ensuing immune response. It remains to be determined whether this inhibitory role is beneficial to the host during as production of TGF-β has been shown to enhance mycobacterial replication (Toossi and Ellner 1998)

1.7 Adaptive Immune response

Pathogens have evolved mechanisms to evade recognition and clearance by the innate immune system. Evolution of the adaptive immune response enabled hosts to specifically recognise such pathogens through pathogen-derived peptides and form life-long protection against previously encountered pathogens.

Control of infection with *M. tuberculosis* and *M. bovis* requires the development of a Th1-type adaptive immune response. Studies in cattle, humans and mice demonstrate

that both CD4 and CD8 T cells subsets are involved in immunity to mycobacteria. The interaction of these T cells with infected Mϕ is central to the outcome of infection and it is likely that the relative importance of the different T cells subsets will depend upon the phase of infection. In brief, infection with mycobacteria is defined by an initial phase which is primarily controlled by innate immune effector cells such as NK cells and γδ T cells. This is followed by acute infection during which adaptive immunity develops to control the rapidly dividing bacilli which is followed by the chronic phase of infection in which the growth of *M. tuberculosis* is stabilised by the adaptive immune response and an equilibrium is established between the mycobacteria and the host's immune system. Failure to control the infection during the acute or latent phase will lead to progressive disease.

The following sections will deal with the role of CD4 and CD8 T cells in immunity to TB summarising the main findings of studies in mouse models, human beings and cattle. Much of the information available regarding immune responses to pathogenic mycobacteria has been reported in *M. tuberculosis* infection of human beings and transgenic mice. Present knowledge of immune responses in bovine TB is far behind that in human TB, therefore the role of CD4 and CD8 T cells in bovine TB will be included where the information is available.

1.7.1 Role of $CD4^+$ T cells

M. tuberculosis and *M. bovis* reside primarily in phagosomal vacuoles within Mϕ therefore mycobacterial antigens will be predominantly processed and presented on MHC class II molecules to $CD4^+$ T cells.

Studies in mouse models of TB

Studies in mice have shown that $CD4^+$ T cells play a central role in the protection against *M. tuberculosis* infection. Early studies showed that adoptive transfer of *M. tuberculosis* specific $CD4^+$ T cells conferred protection and antibody depletion of $CD4^+$ T cells in *M. tuberculosis* infected mice led to an increased bacterial burden and shortened survival times (Orme and Collins 1983; Orme and Collins 1984; Muller,

Cobbold et al. 1987; Pedrazzini, Hug et al. 1987; Leveton, Barnass et al. 1989). These results were further substantiated by experiments performed in mice lacking genes for CD4 and MHC class II molecules as these mice were found to be extremely susceptible to *M. tuberculosis* infection (Ladel, Daugelat et al. 1995; Tascon, Stavropoulos et al. 1998; Caruso, Serbina et al. 1999).

The predominant population of T cells in the granulomas formed in the lungs of *M. tuberculosis*-infected mice are $CD4^+$ T cells (Gonzalez-Juarrero, Turner et al. 2001). The primary effector function of *M. tuberculosis* specific $CD4^+$ T cells is thought to be the production of IFN-γ as the levels of this cytokine are severely diminished early in the infection process in mice deficient in CD4 or MHC Class II (Caruso, Serbina et al. 1999). The early production of IFN-γ by $CD4^+$ T cells may be important in the activation of anti-microbial functions of Mϕ as NOS2 expression was also delayed in the CD4-deficent mice. The IFN-γ and NOS levels in the CD4 and MHC II KO mice were reported to have increased to levels similar to wt mice by 3 wks post-infection and yet these KO mice succumbed prematurely to infection. Thus $CD4^+$ T cells may exert other effector functions that are important to the control of *M. tuberculosis*. It is also possible that the delay in IFN-γ production in the CD4 deficient mice may have profound consequences on the ability of Mϕ to control *M. tuberculosis* replication that cannot be reversed by any amount of IFN-γ produced by $CD8^+$T cells. Furthermore $CD4^+$ T cells were also shown to be important in the recruitment of cytotoxic $CD8^+$ T cells in the lungs of *M. tuberculosis* infected mice which may contribute to the increased susceptibility of mice deficient in functional $CD4^+$ T cells (Serbina, Lazarevic et al. 2001).

$CD4^+$ T cells play a key role in the early and chronic stages of *M. tuberculosis* infection as antibody depletion of $CD4^+$ T cells in chronically *M. tuberculosis* infected mice lead to a rapid reactivation of infection and subsequent death of the mice. The effect of the depletion was not due to lack of IFN-γ or iNOS production as the levels were similar in the control and depleted animals (Scanga, Mohan et al. 2000). These findings indicate that the $CD4^+$ T cells may contribute to the control of *M. tuberculosis* via a mechanism other than production of IFN-γ. One such mechanism may be the killing of infected cells as $CD4^+$ T cells from *M. tuberculosis* infected mice were shown to lyse *M. tuberculosis* infected Mϕ through the binding of FAS-FASL and exocytosis of

cytotoxic granules. However only the $CD4^+$ T cell that lysed infected Mϕ through the exocytosis of cytotoxic granules were able to inhibit *M. tuberculosis* growth (Silva and Lowrie 2000).

$CD4^+$ T cells may also contribute to protective immune response against *M. tuberculosis* through the conditioning of APC. The importance of $CD4^+$ T cells in the priming and maintenance of $CD8^+$ T cells in viral models has been proposed to be dependent on the interaction of CD40 on $CD4^+$ T cells with CD40L expressed by APCs. CD4 priming of APCs can enhance antigen presentation, co-stimulatory activity and cytokine production by the APC (Kalams and Walker 1998; Andreasen, Christensen et al. 2000; Clarke 2000). The role of $CD4^+$ T cell in providing help to $CD8^+$ T cells was demonstrated in CD4 deficient mice in which a delay was observed in the development of *M. tuberculosis* specific $CD8^+$ T cells and the $CD8^+$ T cells recruited to the site of infection in the lung lacked cytotoxic function (Serbina, Lazarevic et al. 2001; Wang, Santosuosso et al. 2004).

Studies in human TB

In human TB, $CD4^+$ T cells are essential to the control of *M. tuberculosis* infection. Direct clinical evidence for this has come from HIV patients who have depressed numbers of functional $CD4^+$ T cells. HIV patients have an increased susceptibility to progressive primary *M. tuberculosis* infection, an increased incidence of reactivation of endogenous *M. tuberculosis* infection and re-infection with *M. tuberculosis* (Barnes, Bloch et al. 1991; Hopewell 1992).

A number of *in vitro* studies have shown that human *M. tuberculosis* specific $CD4^+$ T cells secrete cytokines (IFN-γ and TNF-α), lyse *M. tuberculosis*-infected Mϕ and inhibit *M. tuberculosis* growth (Hansen, Petersen et al. 1987; Ottenhoff, Ab et al. 1988; Hancock, Cohn et al. 1989; Kumararatne, Pithie et al. 1990; Boom, Wallis et al. 1991; Haanen, de Waal Malefijt et al. 1991; Tsukaguchi, Balaji et al. 1995); (Mustafa, Kvalheim et al. 1986; Tsukaguchi, de Lange et al. 1999; Canaday, Wilkinson et al. 2001). The mechanisms by which $CD4^+$ T cells exert their anti-mycobacterial effects has been the source of much debate. Activation of *M. tuberculosis* specific $CD4^+$ T cells induces expression of perforin, granzymes, granulysin and FASL (Canaday,

Wilkinson et al. 2001). Lysis of *M. tuberculosis* infected monocytes by $CD4^+$ T cells was only slightly decreased by blocking FAS but substantially decreased by inhibiting perforin activity. It was also reported that the $CD4^+$ T cell mediated inhibition of *M. tuberculosis* growth was not affected by blocking either perforin or FASL (Canaday, Wilkinson et al. 2001). These findings indicate that $CD4^+$ T cells lyse *M. tuberculosis* infected Mϕ predominantly through granule exocytosis whereas inhibition of *M. tuberculosis* growth is mediated by a mechanism independent of perforin or FASL. Canaday et al (2001) noted that the *M. tuberculosis*-infected targets express very low levels of FAS and therefore the contribution of the FAS-induced pathway in the killing of *M. tuberculosis*-infected cells may not have been fully realised. Previously Oddo and co-workers reported that the induction of apoptosis of infected Mϕ via the FAS-FASL pathway substantially reduced the viability of virulent *M. tuberculosis* (Oddo, Renno et al. 1998).

Therefore it is possible that *M. tuberculosis* specific $CD4^+$ T cells can kill *M. tuberculosis* infected Mϕ via a number of mechanisms which may or may not in turn lead to the killing of *M. tuberculosis*. The ability of $CD4^+$ T cells to lyse *M. tuberculosis* infected Mϕ may also depend upon the stage of infection as $CD4^+$ T cells were shown to not efficiently recognise and respond to heavily infected cells (Lewinsohn, Heinzel et al. 2003). This is likely to be due to the down-regulation of expression of MHC class II molecules on *M. tuberculosis* infected Mϕ (Mazzaccaro, Gedde et al. 1996; Hmama, Gabathuler et al. 1998). In contrast, *M. tuberculosis* infection of DC does not result in diminished expression of MHC class II molecules on the surface of the cells. Therefore priming of $CD4^+$ T cells by *M. tuberculosis* infected DC may be central to the development of $CD4^+$ T cell responses during the *M. tuberculosis* infection. However these activated $CD4^+$ T cells are not stimulated by heavily infected Mϕ because of the lack of MHC class II expression which may in part reflect why the host's immune system is unable to eliminate the mycobacteria.

In humans, *M. tuberculosis* reactive $CD4^+$ T cells recognise and respond to *in vitro* stimulation with PPD, live and dead mycobacterium (Esin, Batoni et al. 1996; Turner and Dockrell 1996). This indicates that *M. tuberculosis* specific $CD4^+$ T cells recognise a large repertoire of *M. tuberculosis* antigens (Havlir, Wallis et al. 1991; Schoel, Gulle

et al. 1992). The most commonly recognised antigens by $CD4^+$ T cells from PPD positive individuals include the 3 proteins that constitute the Ag85 complex (mycolyl transferases involved in the cell wall synthesis), ESAT-6, CFP-10, the 19 kDa and 38 kDa lipoproteins, the 32 kDa and 39 kDa recently identified mycobacterial proteins (Harris, Vordermeier et al. 1993; Surcel, Troye-Blomberg et al. 1994; Dillon, Alderson et al. 1999; Skeiky, Lodes et al. 1999); (Wiker and Harboe 1992; Alderson, Bement et al. 2000). An increased understanding and definition of the *M. tuberculosis* antigens recognised by $CD4^+$ T cells from populations of people with different susceptibilities to infection may highlight which are the immunodominant antigens critical to the development of protective $CD4^+$ T cell responses.

Studies in bovine TB

In cattle, BCG vaccination induces a degree of protection against development of severe disease. Immunohistological comparison of granulomas induced by *M. bovis* infection in BCG-vaccinated and non-vaccinated animals demonstrated that a greater number of $CD3^+$ T cells (IFN-γ^+) but fewer $WC1^+\gamma\delta$ T cells were present in the granulomas of vaccinated animals (Johnson, Gough et al. 2006). This suggests that the protection afforded by BCG-vaccination may be attributed to an enhanced number of mycobacterial reactive IFN-γ producing T cells.

Mycobacteria-reactive $CD4^+$ T cells from BCG-vaccinated animals have been shown to proliferate and produce IFN-γ in response to BCG-infected and PPD-B-pulsed DC (Hope, Kwong et al. 2000). In addition, activation of $CD4^+$ T cells from BCG vaccinated neonates with PPD-B resulted in an up-regulated expression of the α-chain of the IL-2 receptor (CD25). A protective role for $CD4^+$ T cells in cattle was inferred by the finding that a high level of immunity against *M. bovis* infection was observed in BCG-vaccinated animals that generated a strong $CD4^+$ T cell responses to mycobacteria (Buddle, Wedlock et al. 2003).

The levels of $CD4^+$ T cells in blood from *M. bovis* infected animals were shown to increase at 3-4 wks post infection and decrease at a later stage of infection. A similar increase in the percentage of cells expressing CD25 was also reported (Pollock, Pollock

et al. 1996). These observations suggest that in response to infection there is an increase in the proportion of activated $CD4^+$ T cells in the circulating lymphocyte pool which may be due to migration of T cells to the site of infection. A recent study showed that in response to culture with PPD-B, $CD4^+$ T cells from *M. bovis* infected animals up-regulated expression of CD25 and CD44 but down-regulated expression of CD62L (Waters, Rahner et al. 2003). Expression of CD62L mediates adhesion to peripheral lymph node addressins whereas CD44 expression is thought to promote movement through extracellular matrix. Therefore the results indicate that the mycobacterial reactive $CD4^+$ T cells upon activation are more likely to extravasate into inflamed tissue rather than migrate to lymphoid tissue.

Mycobacteria-reactive $CD4^+$ T cells in PBMC from *M. bovis* infected cattle produce IFN-γ in response to culture with PPD, live or dead BCG. A dual role for $CD4^+$ T cells in immunity to *M. bovis* infection was indicated by the fact that depletion of $CD4^+$ T cells from PBMC abrogated the IFN-γ production by both the $CD4^+$ and $CD8^+$ T cells. Additionally $CD4^+$ T cells responded to PPD-pulsed and BCG-infected APC whereas sorted $CD8^+$ required the addition of exogenous IL-2 (Walravens, Wellemans et al. 2002). Therefore $CD4^+$ T cells induced by *M. bovis* infection may act as effector T cells through the production of IFN-γ and as helper cells through the production of IL-2 which may be required for the generation of $CD8^+$ T cell responses.

In addition to production of IFN-γ, $CD4^+$ T cells from *M. bovis* infected animals inhibited the metabolic activity of *M. bovis* when cultured with infected Mϕ (Liebana, Aranaz et al. 2000; Denis, Wedlock et al. 2004), suggesting that *M. bovis* reactive $CD4^+$ T cells may also be mycobactericidal.

1.7.2 Role of $CD8^+$ T cells in TB of mice, humans and cattle

Originally, cellular immunity to mycobacteria was thought to be mediated primarily by IFN-γ producing $CD4^+$ T cells. However, there is an accumulating body of evidence from both human and mouse studies demonstrating the involvement of $CD8^+$ T cells in the control of mycobacterial infection.

1.7.2.1 Evidence for the involvement of classically restricted CD8$^+$ T cells in TB

Initial experiments in mice using adoptive transfer or antibody depletion to investigate the function of CD8$^+$ T cells reported contradictory results. Adoptive transfer of T cells from immune to naïve animals provided a level of protection against infection with *M. tuberculosis* which was abrogated by depletion of CD8$^+$ T cells. Interestingly the CD8-depleted mice maintain reactivity to the footpad test, suggesting that CD4$^+$ T cells mediate this response (Orme and Collins 1983; Orme and Collins 1984). Most studies reported that both CD4$^+$ and CD8$^+$ T cells are required for the control of *M. tuberculosis* infection; (Muller, Cobbold et al. 1987; Orme 1987). CD8$^+$ T cells present in *M. tuberculosis* infected mice were shown to kill *M. tuberculosis*-infected Mϕ and inhibit *M. tuberculosis* growth (De Libero, Flesch et al. 1988; Kaufmann 1988). In contrast, other labs reported that CD8$^+$ T cells were not required for immunity to mycobacteria in mice (Leveton, Barnass et al. 1989; Flory, Hubbard et al. 1992). Depletion of CD8$^+$ T cells was shown not to have a significant effect on the growth of BCG as CD4$^+$ T cells were demonstrated to mediate protective responses against BCG (Pedrazzini, Hug et al. 1987). In addition, Leveton et al reported that whereas animals depleted of CD4$^+$ T cells were more susceptibility to infection with *M. tuberculosis*, depletion of CD8$^+$ T cells had no effect on the survival rates. In fact depletion of CD8$^+$ T cells correlated with lower bacterial numbers and less immunopathology.

Much of the confusion about the significance of CD8$^+$ T cells in immunity to mycobacterial infections in mice may have been due to the different study conditions. Variables such as the use of *M. tuberculosis* or BCG, route of infection, dose administered and the time post-infection at which the response is measured would have influenced the results. From these early studies it appears that CD8$^+$ T cells are not required for the control of BCG but are required for the control of *M. tuberculosis* in mice. More recently CD8$^+$ T cells were shown in mice to be more effective in the control of latent stage of infection rather than acute infection whereas the opposite was true for CD4$^+$ T cells (van Pinxteren, Cassidy et al. 2000).

Studies using knockout (KO) mice that lack genes encoding components of the MHC class I machinery further substantiated an essential role for CD8$^+$ T cells in immunity

to *M. tuberculosis*. One of the most confounding studies was performed in mice lacking the gene for β2-microglobulin (β2-m) which together with the MHC class I α-chain forms the MHC class I molecules expressed on the cell surface. It was demonstrated that mice deficient in this molecule lack $CD8^+$ T cells resulting from the absence of positive selection. The β2-m KO mice quickly succumbed to infection with *M. tuberculosis*. These mice were unable to control *M. tuberculosis* replication as they have a higher bacterial burden compared to wt mice with increases in bacterial load being observed in the liver, lung and spleen. However, no effect was observed in the ability of the β2-m KO mice to control BCG infection (Flynn, Goldstein et al. 1992). This indicates that in mice, $CD8^+$ T cells are required for the control of *M. tuberculosis* infection but not BCG. Immunisation with BCG slightly increased the survival rates of the β2-m KO mice but the animals still succumbed more quickly than wt mice suggesting that the memory $CD4^+$ T cell response induced by vaccination is not sufficient to control *M. tuberculosis* infection.

In contrast to the findings of Flynn and co-workers, Ladel *et al* reported that MHC class I KO mice were more susceptible than control mice to infection with a high dose of BCG (Ladel, Daugelat et al. 1995). These studies indicate that $CD8^+$ T cells are essential in control of *M. tuberculosis* but the importance of these cells in the control of BCG is dose-dependent.

One drawback of using the β2-m KO mice to investigate the role of $CD8^+$ T cells in *M. tuberculosis* infection is that this molecule is also involved in iron metabolism. Iron chelation therapy reduced the susceptibility of β2-m KO mice to infection with *M. tuberculosis* (Schaible, Collins et al. 2002). Furthermore the β2-m KO mice are deficient in both classical class Ia and non-classical class Ib MHC molecules thereby it is not possible to determine what the relative contribution of class Ia and Ib restricted $CD8^+$ T cells in immunity to infection in these KO mice.

Nevertheless classically restricted $CD8^+$ T cells were shown to play an important role in *M. tuberculosis* infection in mice lacking the gene for the transporter associated with antigen presentation (TAP1) which is specific to the MHC class Ia processing and presentation pathway. TAP1 is essential for the loading of peptides onto classical MHC class I molecules. TAP1 KO mice lack classical MHC class I restricted T cell responses and are more susceptible to intravenous challenge with *M. tuberculosis* compared to wt

mice as shown by reduced survival rates and an increased bacterial burden (Behar, Dascher et al. 1999). However, TAP1 KO mice were not as susceptible as β2-m KO mice and this difference may be due to the deficiency in iron metabolism or the contribution of non-classically MHC class Ib restricted $CD8^+$ T cells. Non-classical MHC class I molecules have been shown to present mycobacterial antigens to $CD8^+$ T cells these include HLA-E in humans, H2-M3 in mice and CD1 in humans and mice. These molecules utilize β2-microglobulin and are mostly TAP-independent, therefore the effects observed in the TAP KO mice is mainly attributed to the absence of classically restricted $CD8^+$ T cells.

Sousa et al carried out a comparison of susceptibility of different KO mice to infection with *M. tuberculosis*. The mice were inoculated iv with either high or low doses of *M. tuberculosis* (ranging from 10^6-10^5) (Sousa, Mazzaccaro et al. 2000) and the trend in susceptibility to infection as shown by decreased survival rates was found to be β2-microglobulin>TAP-/->CD8a-/->Perforin-/->wt mice. No difference in susceptibility to infection was observed in the CD1d KO mice compared to wt mice. This suggests that CD1d does not play an essential part in protection against *M. tuberculosis* infection in mice. In addition Urdahl et al demonstrated that mice deficient in classical MHC class Ia molecules defined as $K^{b-/-}D^{b-/-}$ were significantly more susceptible to disease than wt mice but slightly more resistant than β2m KO mice (Urdahl, Liggitt et al. 2003).

There is ample data from mouse models of TB to demonstrate that $CD8^+$ T cells are an important part of the protective immune response against *M. tuberculosis* infection. Furthermore vaccines which elicit $CD8^+$ T cell responses are more protective as animals immunised with DC pulsed with both $CD4^+$ and $CD8^+$ T cell epitopes from Ag85A were protected against challenge with *M. tuberculosis*, whereas no protection was observed with either epitope singularly (McShane, Behboudi et al. 2002).

Studies in Humans - Classically restricted $CD8^+$ T cells

Mycobacteria-reactive $CD8^+$ T cells have been described in BCG vaccinated humans. From *in vitro* studies these cells have been shown to express effector functions demonstrating that they may contribute to immunity to mycobacteria. $CD8^+$ T cells from BCG-vaccinated subjects respond to *in vitro* stimulation with BCG, mycobacterial

antigens Ag85A, Ag85B, 19kDa protein or 38-kDa protein and to a lesser extent PPD-B (Turner and Dockrell 1996; Smith, Malin et al. 1999). The use of metabolic inhibitors indicated that BCG is being presented on MHC class I molecules to these $CD8^+$ T cells as recognition was dependent upon phagocytosis, proteosome activity and Golgi-ER transport (Smith, Malin et al. 1999). $CD8^+$ T cells generated by BCG vaccination also respond to *M. tuberculosis* and *M. avium* infected monocytes. Esin and co-workers reported that $CD8^+$ T cells proliferated in response to live *M. tuberculosis* or *M. avium* but not to killed *M. tuberculosis* or *M. tuberculosis* sonic extract unlike the $CD4^+$ and $\gamma\delta$ T cells respectively (Esin, Batoni et al. 1996). These studies show that $CD8^+$ T cells are likely to be induced by BCG vaccination and that some of these cells respond to antigens that are conserved between BCG, *M. tuberculosis* and *M. avium.*

In healthy PPD-positive subjects *M. tuberculosis* reactive $CD8^+$ T cells can be isolated from the blood and lungs (Tan, Canaday et al. 1997), suggesting that $CD8^+$ T cells are induced by *M. tuberculosis* infection *M. tuberculosis* specific $CD8^+$ T cells in blood from TB patients recognise ESAT-6 peptides presented on classical MHC class I molecules. The ESAT-6 specific $CD8^+$ T cells were present in patients with active TB, after clinical recovery and in healthy contacts (Lalvani, Brookes et al. 1998). These $CD8^+$ T cells were functional and rapidly expressed effector functions after *in vitro* stimulation as shown by production of IFN-γ and lysis of peptide pulsed target cells. The induction of ESAT-6 specific $CD8^+$ T cells during *M. tuberculosis* infection was also reported by Smith and Klein who showed that $CD8^+$ T cells from *M. tuberculosis* infected patients responded highly to ESAT-6 and Ag85 whereas $CD8^+$ T cells from healthy BCG-vaccinated individuals responded to Ag85 but not to ESAT-6 (Smith, Malin et al. 1999; Smith, Brookes et al. 2000). These results show that $CD8^+$ T cell responses are generated during *M. tuberculosis* infection of human beings. A protective role for these $CD8^+$ T cells in the control of *M. tuberculosis* infection has been shown in patients with active TB disease as $CD8^+$ T cells from these individuals produce reduced levels of IFN-γ and display decreased CTL activity but an increase in IL-4 production compared to healthy PPD positive patients (Shams, Wizel et al. 2001; Smith, Klein et al. 2002). Furthermore TB patients with active pulmonary disease had decreased numbers of $CD4^+$ and $CD8^+$ T cells in blood which increased after 6 mths of

successful antibiotic treatment. An increased percentage of $CD8^+$ T cells present in the untreated TB patients expressed the activation marker CD38 indicating that activation of $CD8^+$ T cells occurs during active *M. tuberculosis* infection (Rodrigues, Medeiros et al. 2002).

M. tuberculosis reactive $CD8^+$ T cells in humans predominantly respond to secreted mycobacterial antigens. Epitope mapping studies have identified HLA-A2 restricted epitopes that are present in; ESAT-6, Ag85A and B, the 19k Da lipoprotein, 16k Da protein and CFP-10 (Mohagheghpour, Gammon et al. 1998; Geluk, van Meijgaarden et al. 2000; Caccamo, Milano et al. 2002); (Lalvani, Brookes et al. 1998; Smith, Brookes et al. 2000). In BCG-vaccinated individuals, HLA-B*35 restricted $CD8^+$ T cell epitopes have been identified in *M. tuberculosis* Rv2903c and Ag85 complex (Klein, Smith et al. 2001); (Klein, Hammond et al. 2002) In healthy PPD- positive individuals HLA-B44 and HLA-B14 restricted $CD8^+$ T cells clones that recognise CFP-10 have been isolated (Lewinsohn, Zhu et al. 2001). Recently, CFP-10 was shown to contain epitopes that are expressed on both common MHC class I and II haplotypes (Shams, Klucar et al. 2004). The ability of CFP-10 to elicit both $CD8^+$ and $CD4^+$ T cells suggests that this antigen may be a prime candidate for inclusion in new TB vaccines or in diagnostic tests.

The identification of a number of epitopes recognised by $CD8^+$ T cells induced by vaccination or *M. tuberculosis* infection demonstrates the involvement of these cells in the immune response elicited to mycobacteria in humans.

In cattle, the analysis of immune responses induced by vaccination or infection are predominantly performed on whole blood or PBMC and the responding cells are rarely defined. Therefore the role of $CD8^+$ T cells in bovine TB has yet to be clearly addressed. BCG vaccination of cattle provides partial protection against *M. bovis* challenge, in that vaccinated animals have significantly less bacterial burden and a decreased lesion score compared to non-vaccinated animals. Therefore it is likely that BCG induces memory T cells that are able to control but not clear the *M. bovis*.

In animals vaccinated with BCG, both BCG-reactive $CD4^+$ and $CD8^+$ cells have been identified. These cells can be stimulated to proliferate and produce IFN-γ upon *in vitro*

culture with BCG-infected DC. One of the main differences between cattle and humans is that the γδ T cells in cattle account for a greater percentage of the major circulating lymphocyte pool. Two populations of bovine γδ T cells have been identified in bovine blood based on expression of the CD8 co-receptor and the scavenger receptor WC1, expression of these is mutually exclusive (MacHugh, Mburu et al. 1997). It was demonstrated in the study by Hope et al that 53% of the BCG-reactive $CD8^+$ cells in BCG-vaccinated animals expressed the γδ T cell receptor (TCR). Therefore it is possible that the response measured may have been due to the expansion of BCG-reactive $CD8^+$ αβ or γδ T cells or a combination of the two subtypes (Hope, Kwong et al. 2000).

A population of $CD8^+$ cells from non-vaccinated neonatal calves were found to respond to BCG-infected DCs. Phenotypic analysis showed that the responding cells were both $CD8^+CD3^-$ and $CD8^+CD3^+$. Further analysis showed that the $CD8^+CD3^+$ cells were γδ T cells and the $CD8^+CD3^-$ T cells were most likely to be NK cells (Hope JC et al 2002). Taracha and co-workers demonstrated that $CD8^+$ cells from cattle primed with fowlpox virus expressing Ag85A and boosted a number of times with MVA-Ag85A, produced IFN-γ in response to culture with Ag85A peptides (Taracha, Bishop et al. 2003). The peptides used in this study were 20 amino acids (aa) in length and no APC was added to the culture therefore in this system, the $CD8^+$ cells must have been processing the peptide and presenting it to each other. These results suggest that it is possible to induce mycobacterial reactive $CD8^+$ cells by vaccination with BCG or recombinant viruses expressing Ag85A.

In cattle experimentally infected with *M. bovis* changes in the circulating T cell subpopulations have been described. It was reported that in the latter stages of infection the CD4:CD8 ratio decreased to levels below that observed prior to infection. It was proposed that this was due to an increase in the percentage of $CD8^+$ cells. It was also reported that T cell clones reactive to PPD-B isolated at the early stages of infection were mostly $CD4^+$ and γδ T cells, whereas PPD-B reactive clones isolated at the latter stages of infection predominantly expressed CD8 (Pollock, Pollock et al. 1996). The true reactivity of $CD8^+$ T cells in the Pollock report may not have been recognised as PPD-B was used to stimulate the cells whereas different results may have been obtained if live mycobacteria had been used.

In addition, $CD8^+$ cells have been proposed to participate in the DTH tuberculin reaction of *M. bovis* infected cattle as an increase in the trafficking of $CD8^+$ cells was observed at the site of PPD injection (Doherty, Bassett et al. 1996).

One of the first studies to specifically investigate $CD8^+$ cell responses in infected cattle was performed by Liebana et al. It was reported that $CD8^+$ cells from *M. bovis* infected animals, proliferated and produced IFN-γ in response to stimulation with *M. bovis*, PPD-B and *M. bovis* sonic extract (MBSE). The use of metabolic inhibitors CYT-D and BFA indicated that recognition of both the live mycobacteria and the exogenously added antigens required the endogenous processing pathway, suggesting that these antigens were presented on MHC class I molecules to the $CD8^+$ cells (Liébana, Girvin et al. 1999).

A recent report showed that $CD8^+$ T cells present in PBMC from *M. bovis* infected animals produce IFN-γ after culture with PPD-B, killed and live BCG. However, depletion of $CD4^+$ T cells from the PBMC abrogated this $CD8^+$ T cell response. Furthermore $CD8^+$ cells isolated from *M. bovis* infected animals required addition of IL-2 in order to produce IFN-γ in response to BCG-infected or PPD-B pulsed APCs (Walravens, Wellemans et al. 2002). This is in contrast to results of Liebana *et al* showing that sorted $CD8^+$ cells responded to PPD-B, MBSE and *M. bovis* without the need for exogenous IL-2. The difference between the two studies may be attributed to different culture periods as in the study by Walravens et al, the sorted $CD8^+$ cells were cultured for only 24 hrs whereas in the study by Liebana et al the $CD8^+$ cells were cultured for 5 days. Moreover, Liebana et al used *M. bovis* or *M. bovis* antigens to stimulate the cells which may induce greater $CD8^+$ T cell responses as they contains more antigens than the BCG used by Walravens et al. Additionally $CD8^+$ cells induced by *M. bovis* infection of cattle may be specific for antigens that are absent in BCG.
It has been proposed that $CD8^+$ cells may play a role in the immunopathology observed in bovine TB as it was shown that cattle depleted of $CD8^+$ cells within the first two weeks of *M. bovis* infection had decreased lesion scores compared to control animals. The same study showed that the CD8 depleted animals also produced less IFN-γ in response to PPD-B stimulation of whole blood (Villarreal-Ramos, McAulay et al.

2003). The timeframe of the depletion study suggests that the observed effects may be due to the removal of innate immune cells that express $CD8^+$ such as NK cells or γδ T cells as previous reports have implicated $CD8^+$ T cells in the control of latter stages of infection (Pollock, Pollock et al. 1996).

1.7.2.2 Non-classically restricted $CD8^+$ T cells

In addition to MHC class I restricted $CD8^+$ T cells, a role for CD1-restricted $CD8^+$ T cells has been proposed in human TB. CD1 molecules are non-polymorphic, MHC class I-like molecules and associate with β2-microglobulin. They are expressed on the cell surface and acquire antigen present in endosomal and phagosomal compartments where phagocytosed mycobacterial antigens are mostly like to traffic through. A population of $CD1^+$ cells were identified in the lymph nodes of TB-infected patients and this molecule was co-expressed by DC-SIGN indicating that CD1 is expressed mainly by DCs *in vivo* (Gansert, Kiessler et al. 2003). Furthermore Mϕ have been shown to either lack expression of CD1 or express low levels of these molecules (Porcelli and Modlin 1999).

The role of CD1-restricted T cells in mice is unclear as no increase in susceptibility to infection with *M. tuberculosis* was observed in CD1 KO mice (Behar, Dascher et al. 1999). In humans, two populations of CD1-restricted mycobacterial reactive αβ T cells have been isolated from TB infected individuals. The majority of CD1-restricted T cells were found to be $CD8^-CD4^-$ with a small population being $CD8^+CD4^-$ (Porcelli, Morita et al. 1992; Beckman, Porcelli et al. 1994; Sieling, Chatterjee et al. 1995; Moody, Guy et al. 2000; Moody, Ulrichs et al. 2000; Ulrichs and Porcelli 2000); (Porcelli and Modlin 1999). Moreover CD1-restricted $CD8^+$ T cells have been isolated from BCG–vaccinated individuals (Lewinsohn, Alderson et al. 1998; Lewinsohn, Briden et al. 2000; Kawashima, Norose et al. 2003). These T cells recognise mycobacterial lipid and lipoglycan antigens such as mycolic acids and phosphatidyl-inositol-mannosides.

The presence of other non-classically restricted populations of *M. tuberculosis* reactive $CD8^+$ T cells were shown by the finding that $CD8^+$ T cell clones isolated from two PPD positive healthly humans recognised and responded to *M. tuberculosis*-infected DC

independently of HLA-A, B, C and CD1 expression (Lewinsohn, Alderson et al. 1998). Furthermore in two healthy individuals exposed to *M. tuberculosis*, classically restricted *M. tuberculosis* reactive $CD8^+$ T cells were shown to account for only 4% and 26% of the total $CD8^+$ T cell clones isolated. The remaining *M. tuberculosis* reactive $CD8^+$ T cell clones were non-classically MHC class I restricted but not CD1-restricted (Lewinsohn, Briden et al. 2000).

1.7.2.3 Effector functions of mycobacterial specific $CD8^+$ T cells

There are three main effector functions of $CD8^+$ T cells which could potentially contribute to immunity to mycobacteria. These are the production of cytokines, cytolysis and direct microbicidal activity. The relative importance of each of these effector functions in the control of mycobacteria is unknown but it is likely that it involves a combination of these is required.

Production of cytokines

Studies in mouse models of TB have shown that cytokines associated with Th1-type immune response such as TNF-α and IFN-γ are required for the control of BCG and *M. tuberculosis* infection. These two cytokines can act alone or in synergy to activate the anti-microbicidal effector functions of infected murine Mϕ.

Mice deficient in the IFN- receptor (R) are unable to control replication of BCG or *M. tuberculosis* and succumbed quicker to infection than wt mice. Disseminated disease occurs in these IFN-γR KO due to a failure to produce nitric oxide (NOS) and development of irregular granulomas and formation of large areas of tissue necrosis (Cooper, Dalton et al. 1993; Flynn, Chan et al. 1993; Kamijo, Le et al. 1993).

The production of IFN-γ by *M. tuberculosis* specific $CD8^+$ T cells in mice is required for the control of *M. tuberculosis* replication but it is not sufficient to protect against primary infection (Tascon, Stavropoulos et al. 1998; Caruso, Serbina et al. 1999). In CD4 KO mice an increased number of IFN-γ secreting $CD8^+$ T cells were found to be present in the lung and the percentage of IFN-γ expressing T cells were similar between wt and CD4 KO mice. Although these mice still succumbed prematurely to infection, these results indicate that $CD8^+$ T cells are able to compensate for IFN-γ produced by

$CD4^+$ T cells in the wt mice. The inability of $CD8^+$ T cells to protect mice against primary *M. tuberculosis* infection in the CD4 KO mice may be explained by the finding that IFN-γ production by $CD8^+$ T cells is independent of $CD4^+$ T cells whereas cytotoxicity of the *M. tuberculosis* specific $CD8^+$ T cells in the lung but not in the draining lymph node was impaired in CD4 KO mice (Serbina, Lazarevic et al. 2001). However, in some experimental settings IFN-γ producing $CD8^+$ T cells are able to protect mice against *M. tuberculosis* infection. BCG vaccination of CD4 KO mice with BCG induced IFN-γ producing $CD8^+$ T cells which were able to control a subsequent challenge with *M. tuberculosis* when inoculated at 6 or 12 wks post-vaccination but not at 3 wks post-vaccination. This delay in protection was attributed to a delay in the distribution and recruitment of the $CD8^+$ T cells in the CD4 KO mice to the spleen and lung in comparison to wt mice (Wang, Santosuosso et al. 2004). Furthermore IFN-γ secreting $CD8^+$ T cell have been shown to mediate memory responses in the lungs of mice following secondary challenge with *M. tuberculosis* (Serbina and Flynn 2001).

In addition to IFN-γ, TNF-α plays a central role in the cellular organisation and development of the granuloma (Kindler, Sappino et al. 1989). Mice deficient in the TNF-a receptor or depleted of TNF-α as these mice display a decreased production of RNI by infected Mϕ, contain a greater level of necrosis and subsequently succumb more quickly to *M. tuberculosis* infection compared to wt mice. (Flynn, Goldstein et al. 1995).

In vitro studies of TB in human beings have demonstrated that $CD8^+$ T cells from BCG vaccinated and healthy PPD positive patients produce IFN-γ and TNF-α in response to a number of mycobacterial antigens (Lalvani, Brookes et al. 1998; Lewinsohn, Alderson et al. 1998; Canaday, Ziebold et al. 1999; Smith, Malin et al. 1999; Smith, Brookes et al. 2000). The importance of IFN-γ in immunity to mycobacteria in humans is demonstrated in patients with mutations in the IFN-γ R as these individuals have a significantly increased susceptibility to mycobacterial infections (Jouanguy, Altare et al. 1997; Jouanguy, Doffinger et al. 1999; Jouanguy, Lamhamedi-Cherradi et al. 1999; Dorman and Holland 2000; Sasaki, Nomura et al. 2002). The control of latent TB in humans was demonstrated to be dependent upon the production of TNF-α by the development of active TB in patients receiving anti-TNF-α therapy for Rheumatoid

arthritis and Crohns disease (Keane, Gershon et al. 2001).Moreover $CD8^+$ T cells from TB patients with active pulmonary disease produce less IFN-γ and TNF-α than healthy PPD positive patients (Sodhi, Gong et al. 1997; Hirsch, Toossi et al. 1999; van Crevel, Karyadi et al. 2000; Shams, Wizel et al. 2001; Smith, Klein et al. 2002). Although it is clear that IFN-γ is necessary for the control of mycobacterial infection in humans, unlike in mice, IFN-γ does not efficiently activate *M. tuberculosis* infected human monocytes *in vitro* (Rook, Taverne et al. 1987).

A role for NK cells in the production of IFN-γ from *M. tuberculosis* specific $CD8^+$ T cells was indicated by the finding that depletion of NK cells from PBMC from healthy PPD positive individuals prior to stimulation with *M. tuberculosis* significantly reduced the secretion of IFN-γ by $CD8^+$ T cells (Vankayalapati, Klucar et al. 2004). It was shown that IFN-γ produced by the NK cells induces the production of IL-15 and IL-18 from *M. tuberculosis* infected monocytes which in turn enhances the expansion of IFN-γ secreting *M. tuberculosis* specific $CD8^+$ T cells (Vankayalapati, Wizel et al. 2001; Schluns, Williams et al. 2002; Umemura, Nishimura et al. 2002).

In cattle, $CD8^+$ cells from BCG-vaccinated and *M. bovis* infected animals have been shown to produce IFN-γ in response to stimulation with live mycobacteria and mycobacterial antigens (Liébana, Girvin et al. 1999; Buddle, Wedlock et al. 2003; Vitale, Reale et al. 2006). In addition, a number of heterologous prime-boost vaccination protocols that elicit a high degree of protection against *M. bovis* infection have been shown to induce high levels of IFN-γ (Skinner, Wedlock et al. 2005; Wedlock, Denis et al. 2005). Although the responding cells have not been defined, it is likely that $CD8^+$ cells will have contributed to this IFN-γ production. It was shown by Taracha and collegues that heterologous prime-boosting of cattle with different replication attenuated pox viruses expressing Ag85A induced IFN-γ secreting $CD8^+$ cells (Taracha, Bishop et al. 2003).

Cytolysis

One of the major functions of $CD8^+$ T cells is the killing of target cells. Cytotoxic T cells can induce apoptosis of targets cells via a number of different pathways. One of the main mechanisms investigated is exocytosis of cytotoxic granules containing perforin and granzymes. Human and bovine cytotoxic granules also contain the anti-microbial protein granulysin. Perforin is an essential constituent of the cytotoxic granules as mice deficient in perforin lack the ability to kill target cells via the exocytosis of cytotoxic granules (Kagi, Ledermann et al. 1994). It was originally proposed that perforin functions by forming pore-like structures in the cell membrane allowing the entry of other granule constituents such as granzymes into the target cells. More recent studies have described a role for perforin in the release of granzymes from endosomes into the cytosol of the target cell (Podack, Konigsberg et al. 1985; Podack, Young et al. 1985; Pinkoski, Hobman et al. 1998; Metkar, Wang et al. 2002).

Granzymes are serine proteases that activate caspase-dependent and independent pathways which ultimately culminate in apoptosis of the target cell (Russell and Ley 2002). $CD8^+$ T cells can also induce of apoptosis of target cells via the ligation of FAS on the target cell with FASL on the $CD8^+$ T cell. Activation of the FAS receptor initiates a caspase cascade which culminates in apoptosis of the target cell (Itoh, Yonehara et al. 1991; Oddo, Renno et al. 1998). Studies of mice deficient in FAS, FASL, perforin or granzymes indicate that murine $CD8^+$ T cells predominantly use the granule exocytosis pathway to mediate cytotoxicity whereas the FAS-FASL pathway is the main mechanism by which $CD4^+$ T cells induce cytotoxicity (Kagi, Ledermann et al. 1994; Kagi, Vignaux et al. 1994; Stalder, Hahn et al. 1994).

Other mechanisms by which T cells have been reported to mediate cytoxicity is through the binding of other membrane bound molecules such as death receptors TNFR1 and TNF-related apoptosis-inducing ligand (TRAIL) (Zhang, Hartig et al. 2005). Finally, Lammas and colleagues reported that $CD8^+$ T cells were able to inhibit the growth of BCG contained inside Mϕ through the release of ATP (Lammas, Stober et al. 1997).

It has been demonstrated in both human and murine studies that *M. tuberculosis* reactive $CD8^+$ T cells induced by vaccination and *M. tuberculosis* infection are able to kill *M. tuberculosis* infected macrophages (Turner and Dockrell 1996; Smith, Malin et

al. 1999; Cho, Mehra et al. 2000; Smith, Brookes et al. 2000). This killing of macrophages infected with mycobacteria can result in several scenarios. The first is the killing of both the macrophage and the mycobacteria. The second scenario would the killing of only the Mϕ resulting in release of free mycobacteria into the surrounding environment. The third is that the Mϕ is killed and the mycobacteria are contained in apoptotic blebs.

The release of free mycobacteria or mycobacteria contained in apoptotic blebs is likely to result in the uptake and killing of the mycobacteria by non-infected more proficient phagocytic cells (De Libero, Flesch et al. 1988). It has been shown that apoptosis of infected Mϕ, inhibits the growth and spread of *M. tuberculosis* (Molloy, Laochumroonvorapong et al. 1994; Fratazzi, Arbeit et al. 1999). Furthermore the uptake of apoptotic blebs containing mycobacteria by DCs has been proposed as a mechanism by which mycobacterial antigens could be expressed on either MHC class I or CD1 molecules (Schaible, Winau et al. 2003)

The mechanisms by which *M. tuberculosis* reactive $CD8^+$ T cells kill infected Mϕ and mycobacteria are unclear. *M. tuberculosis* infection of mice generates MHC class I restricted $CD8^+$ T cells that express perforin and lyse *M. tuberculosis* infected Mϕ and are found in lungs and draining lymph nodes (Serbina, Liu et al. 2000). Studies utilising mice deficient in perforin or FAS have shown that a defective FAS-FASL pathway results in higher bacterial loads during *M. tuberculosis* infection compared to mice deficient in perforin (Turner J et al 2001). However, perforin deficient mice show a decreased survival time compared to wt mice (Sousa, Mazzaccaro et al. 2000). In contrast, Laochumroonvorapong et al demonstrated that perforin and FAS-mediated cytotoxicity was not required for the control of *M. tuberculosis* during the early stages of infection (Laochumroonvorapong, Wang et al. 1997). This study did not investigate whether these molecules are required during chronic infection when Mϕ are more likely to be unable to control *M. tuberculosis* growth and harbour a greater number of mycobacteria.

Although deficiencies in either perforin or FAS impart no increase or only a modest increase in susceptibility to *M. tuberculosis* infection in mice, it is possible that the importance of these molecules may vary at different stages of infection. It is also

possible that one pathway is sufficient and can compensate for the lack of the other. Moreover, an unidentified cytotoxic mechanism may be involved which is utilised by $CD8^+$ T cells during *M. tuberculosis* infection of mice as inhibition of *M. tuberculosis* growth was mediated in the absence of perforin and granzymes (Cooper, D'Souza et al. 1997).

The recruitment of cytotoxic $CD8^+$ T cells in the lungs in *M. tuberculosis* infected mice requires $CD4^+$ T cell help as no cytotoxicity was observed by the *M. tuberculosis* specific $CD8^+$ T cells in the lungs of infected mice deficient in $CD4^+$ T cells (Serbina, Lazarevic et al. 2001)
This diminished cytotoxicity of $CD8^+$ T cells was not observed in the draining lymph nodes of CD4 deficient mice but did correlate with a decrease in expression of IL-15 and IL-2 in the lungs. These results indicate that through the production of cytokines IL-2 and IL-15 $CD4^+$ T cells may enhance the maintenance and recruitment of *M. tuberculosis*-specific cytotoxic $CD8^+$ T cells in the lungs of mice.

In human TB, two populations of *M. tuberculosis* reactive cytotoxic $CD8^+$ T cells have been described, one is restricted by classical MHC class I molecules and the other is restricted by CD1. The CD1-restricted $CD8^+$ T cells have been studied by Stenger and co-workers. Their studies have shown that CD1-restricted $CD8^+$ T cells lyse *M. tuberculosis*-infected Mϕ predominantly via the granule exocytosis pathway which is accompanied by inhibition of mycobacterial viability (Stenger, Mazzaccaro et al. 1997). The lysis of target cell and antimicrobial activity towards *M. tuberculosis* was dependent upon release of cytotoxic granules but was independent of caspase activity and caspase induced nuclear cell apoptosis (Thoma-Uszynski, Stenger et al. 2000). These data suggest that CD1-restrcited $CD8^+$ T cells mediate anti-microbial activity towards *M. tuberculosis* by the release of a granule constituent such as granulysin in a process which is separate from the induction of apoptosis of the infected cell.

It was demonstrated by Lalvani and colleagues that HLA-class I restricted $CD8^+$ T cells specific for epitopes in the *M. tuberculosis* protein ESAT-6 isolated from TB patients after clinical recovery and in healthy contacts lysed peptide-pulsed target cells (Lalvani, Brookes et al. 1998). Likewise Cho et al reported that classically restricted

$CD8^+$ T cells exhibited lysed *M. tuberculosis*-infected Mϕ and significantly reduced mycobacterial numbers (Cho, Mehra et al. 2000). These peptide-specific $CD8^+$ T cells express granulysin and perforin which may account for the observed anti-microbial activity. Furthermore, as these $CD8^+$ T cells were isolated from TB patients this shows that the epitopes recognised by these cells are presented during the natural course of infection.

Similarly Canaday and co-workers reported that the lysis of *M. tuberculosis*-infected Mϕ by MHC class I restricted $CD8^+$ T cells from healthy tuberculin positive individuals was predominantly mediated by exocytosis of cytotoxic granules rather than ligation of FAS-FASL. Moreover, inhibition of *M. tuberculosis* growth by *M. tuberculosis*-specific $CD8^+$ T cells did not require perforin or signalling through FAS-FASL (Canaday, Wilkinson et al. 2001).

These results indicate that *M. tuberculosis*-specific $CD8^+$ T cells primarily lyse infected Mϕ via the release cytotoxic granules whereas the inhibition of *M. tuberculosis* growth is likely to be mediated by a granule constituent that does not require perforin to gain access into the target cell. Alternatively, *M. tuberculosis*-specific $CD8^+$ T cells may also utilise some other anti-microbial mechanism such as the release of ATP or binding of membrane bound TNF-α with death receptors on the Mϕ to inhibit *M. tuberculosis* viability.

A recent report demonstrated that a subset of *M. tuberculosis*-reactive $CD8^+$ T cells co-ordinately expressed granulysin, perforin and the chemokine CCL5. It was shown that CCL5 produced by the $CD8^+$ T cells attracted *M. tuberculosis* infected monocytes to migrate towards $CD8^+$ T cells that are armed with cytolytic molecules perforin and granulysin. It is likely that these $CD8^+$ T cells were classically restricted as monocytes which lack expression of CD1 were used to stimulate (Stegelmann, Bastian et al. 2005). Thus, expression of CCL5 may assist the killing of *M. tuberculosis*-infected Mϕ by effector $CD8^+$ T cells.

The relative contribution *in vivo* of CD1-restricted $CD8^+$ T cells and MHC class I restricted $CD8^+$ T cells in the control of *M. tuberculosis* infection is unclear as both

these cell types have been isolated from TB patients and have been reported to produce IFN-γ, lyse infected cells and directly kill mycobacteria.

In *M. bovis* infected cattle, cytotoxic activity against *M. bovis* infected Mϕ was detected in PBMC at 4 wks post-infection. Depletion of either $CD8^+$ or $WC1^+$ cells from the PBMC decreased the level of killing in some animals. The lysis of the *M. bovis* infected Mϕ was accompanied by a decrease in mycobacterial numbers inside the Mϕ (Skinner, Parlane et al. 2003). $CD8^+$ cells isolated from MBSE stimulated PBMC from *M. bovis* infected animals induced the release of metabolically active *M. bovis* from infected Mϕ (Liebana, Aranaz et al. 2000). These finding suggest that the $CD8^+$ cells from *M. bovis* infected animals may have the capacity to lyse infected Mϕ.

Moreover, $CD8^+$ T cells from BCG-vaccinated and *M. bovis* infected animals inhibited the metabolic activity of *M. bovis* inside Mϕ. This inhibition was dependent upon cell-contact, production of IFN-γ and nitric oxide (NO) (Liebana, Aranaz et al. 2000; Denis, Wedlock et al. 2004), indicating that $CD8^+$ T cells from BCG-vaccinated animals inhibit *M. bovis* replication inside Mϕ through the secretion of IFN-γ and the subsequent activation of NO production by the infected Mϕ.

Direct killing of mycobacteria

A mechanism by which cytotoxic $CD8^+$ T cells have been suggested to kill mycobacteria is through the release of granulysin. Granulysin is a potent antimicrobial protein that is present in the cytotoxic granules of human, swine and bovine T cells and NK cells (Andersson, Gunne et al. 1995; Stenger, Hanson et al. 1998; Dieli, Troye-Blomberg et al. 2001). In addition to being cytotoxic to mycobacteria, granulysin has been shown to kill a number of micro-organisms which include gram-positive and gram-negative bacteria, fungi, protozoa, parasites and viruses (Andreu, Carreno et al. 1999; Ernst, Thoma-Uszynski et al. 2000; Krensky 2000; Andra, Berninghausen et al. 2001; Hata, Zerboni et al. 2001; Ma, Spurrell et al. 2002; Jacobs, Bruhn et al. 2003; Endsley, Furrer et al. 2004). Granulysin is active against drug susceptible and importantly drug-resistant strains of *M. tuberculosis* (Stegelmann, Bastian et al. 2005). It has been proposed that granulysin is a lytic molecule and inserts itself into the cell

membrane inducing ion fluxes which lead to the induction of apoptosis (Clayberger and Krensky 2003).

It has been proposed that the inhibition of *M. tuberculosis* growth and viability by both MHC class I restricted and CD1-restricted $CD8^+$ T cells, is mediated by the release of cytotoxic granules containing granulysin (Stenger, Hanson et al. 1998). The lack of a murine homologue of granulysin means that the functional significance of this molecule in the control of *M. tuberculosis* infection has yet to be investigated.

A bovine homologue of granulysin, named Bolysin was first identified by Endsley and co-workers. Expression of this granulysin mRNA was induced in CD4, CD8 and γδ T cells by mitogenic activation. It was also reported that the granulysin was expressed in lymph node lesions from *M. bovis* infected animals (Endsley, Furrer et al. 2004). The role of granulysin in the cytotoxic activity of bovine $CD8^+$ T cells induced by *M. bovis* infection has not yet been addressed.

1.7.2.4 Unique role of $CD8^+$ T cells

Although the effector functions described for $CD8^+$ T cells and $CD4^+$ T cells in the control of *M. tuberculosis* infection, overlap considerably, $CD8^+$ T cells could additionally function to recognise MHC class II negative cells. *M. tuberculosis* has been reported to infect epithelial cells which lack MHC class II but express MHC class I. Furthermore *M. tuberculosis* has been shown to modulate the response of Mϕ, which has been shown to include the down-regulation of expression of MHC class II but not MHC class I molecules. Therefore these cells would escape recognition by $CD4^+$ T cells but not by $CD8^+$ T cells (Mazzaccaro, Gedde et al. 1996). The redundancy of effector functions between $CD4^+$ and $CD8^+$ T cells may be related to the possibility that mobilisation of each of the cell types may occur at different stages of *M. tuberculosis* infection. It is has been postulated that while IFN-γ and TNF-α produced by either $CD4^+$ or $CD8^+$ T cells are sufficient to maintain low numbers of mycobacteria inside Mϕ at early stages of infection. *M. tuberculosis* has been shown to modulate effector function of the host Mϕ in particular it inhibits the ability of Mϕ to respond to IFN-γ and TNF-α. Thus, allowing the *M. tuberculosis* to grow inside unresponsive Mϕ. This increased metabolism and growth is likely to lead to an abundance of *M. tuberculosis* antigens in the cytosol and therefore an increased presentation of *M. tuberculosis*

antigens on MHC class I molecules to $CD8^+$ T cells. It is at this stage of infection that cytotoxic $CD8^+$ T cells may play an essential role, in support of this $CD8^+$ T cells have been shown to preferentially recognise heavily infected cells whereas $CD4^+$ T cells recognised and responded to cells harbouring fewer mycobacteria. Furthermore in *M. tuberculosis*-infected mice IFN-γ-producing $CD4^+$ T cells predominate the immune response during the acute phase of infection whereas IFN-γ producing $CD8^+$ T cells were essential to the control of *M. tuberculosis* during the latent stage of infection in the lung (van Pinxteren, Cassidy et al. 2000).

Differential utilisation of effector functions by $CD4^+$ and $CD8^+$ T cells in TB patients has been demonstrated by Lewinsohn and co-workers who reported that CFP-10 specific $CD4^+$ T cells preferentially produced IFN-γ whereas CFP-10 specific $CD8^+$ T cells preferentially used the granule exocytosis pathway to induce lysis of infected cells (Lewinsohn, Heinzel et al. 2003).

1.7.2.5 Comparisons between human, cattle and mice

Mycobacteria-reactive $CD8^+$ T cells have been identified in mice, humans and cattle. These cells are generated in response to BCG vaccination and infection with either *M. tuberculosis* or *M. bovis*. The kinetics of this response may also be similar as in cattle activation of $CD8^+$ T cells was proposed to occur at later stages of infection and in mice $CD8^+$ T cells were more important in the control of latent infection compared to the early stages of *M. tuberculosis* infection. Mycobacteria-reactive $CD8^+$ T cells have been shown to respond to *in vitro* stimulation with live mycobacteria and derived antigens and similar effector functions of these cells have been described in humans, cattle and mice. In response to stimulation, $CD8^+$ T cells from humans and mice produce cytokines TNF-α and IFN-γ, lyse infected Mϕ and inhibit mycobacterial growth. Although the studies in cattle are limited, mycobacteria-reactive $CD8^+$ T cells have been shown to produce IFN-γ, inhibit *M. bovis* metabolism and induce the release of *M. bovis* from infected Mϕ. On the other hand, significant differences in $CD8^+$ T cell responses are also apparent between mice and humans which include expression of the effector molecule granulysin, which is absent in mice and has been proposed to play a central role in the killing of mycobacteria. A bovine homologue of granulysin has been

defined and is expressed by bovine $CD8^+$ T cells. Therefore cattle provide a model in which to investigate the role of these cells in TB.

Evaluation of some aspects of immune responses in murine models of TB may have limited significance to human and bovine TB as mice are highly susceptible to infection. The pathogenesis and course of TB in humans and cattle are similar. Moreover some vaccines that have shown promise in eliciting protective immune responses in mice have not shown the same promise in human studies. The differences that exist between humans and cattle, and mice may reflect that fact that mice are not a natural host of mycobacteria and eventually succumb to infection. In contrast most humans, similar to cattle, control mycobacterial infection and less than 10% of those infected actually develop active disease. Therefore studies of TB patients when possible are invaluable. However, the testing of the efficacy of new vaccines at providing protection against disease requires an animal model and it is proposed more parallels may be drawn between studies in cattle and humans compared to mice and humans.

1.7.3 MHC class I processing and presentation of mycobacterial antigens

Mycobacteria in particular, *M. tuberculosis* and *M. bovis* primarily infect Mϕ and reside within arrested phagosomes at an early endosomal stage (Sturgill-Koszycki, Schlesinger et al. 1994; Clemens and Horwitz 1995; Clemens, Lee et al. 2000). Antigens contained within these compartments are typically processed by MHC class II pathway, this raises the question of how do antigens from the mycobacteria contained in the phagosomes access the cytosolic MHC class I processing pathway. Several pathways have been proposed, these can be broadly divided into cytosolic and non-cytosolic pathway.

In the cytosolic model mycobacterial antigens inside permeable phagosomes gain access to the cytosol and are processed and presented on MHC class I molecules similar to endogenously derived antigens. Alternatively processing and presentation of mycobacterial antigens by the non-cytosolic pathway is not dependent upon conventional MHC class I processing machinery but antigens are loaded onto MHC class I molecules in the endosomal-lysosomal route.

1.7.3.1 Cytosolic pathways

Previously, a model using OVA albumin demonstrated that exogenous antigens can escape into the cytosol and be processed and presented on MHC class I molecules similar to endogenous antigens (Kovacsovics-Bankowski and Rock 1995). The ability of exogenous antigens to escape into the cytosol was investigated in *M. tuberculosis* infection. It was reported that *M. tuberculosis*- or BCG-infected Mϕ loaded with soluble OVA resulted in a greater expression of OVA peptides on MHC class I molecules compared to uninfected Mϕ loaded with soluble OVA. This process was dependent upon the presence of live mycobacteria and TAP activity (Mazzaccaro, Gedde et al. 1996). To further illustrate the increased permeability of mycobacterial containing phagosomes, Teitelbaum *et al* demonstrated that injection of fluorescent markers up to 70 kDa in size directly into the cytosol of BCG-infected Mϕ, localised to phagosomes containing live but not dead mycobacteria (Teitelbaum, Cammer et al. 1999). Moreover *M. tuberculosis* secreted antigens CFP-10 and ESAT-6 are likely to be processed and presented via the traditional MHC class I pathway as it is dependent on proteasomal degradation and ER-Golgi transport (Grotzke and Lewinsohn 2005). In contrast Schaible and colleagues found that fluorescently or radioactively labelled *M. tuberculosis* antigens were only present in the cytosol at low levels (Schaible, Winau et al. 2003).

Another cytosolic pathway has been recently proposed in which phagosomes act as autonomous organelles capable of cross-presentation. In these studies, phagosomal membranes were proposed to have originated from the ER and contained protein translocation complex Sec61 and molecules involved in MHC class I processing. These phagosomes associated with proteasomes and contain MHC class I:peptide complexes (Gagnon, Duclos et al. 2002; Ackerman, Kyritsis et al. 2003; Desjardins 2003; Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003). In this model the antigens escape from the phagosome into the cytosol and are degraded by proteasomes. The peptides are then transported back into the phagosome and loaded on MHC class I molecules. As trafficking of MHC class I: peptide complexes to the cell surface was only partially inhibited by BFA it has been proposed that this can occur in the absence of ER-Golgi transport. Previously it has been reported that some *M. tuberculosis*-

specific $CD8^+$ T cells recognise *M. tuberculosis* antigens that are processed by a lactacystin-sensitive, cytochalasin D sensitive, BFA insensitive pathway. It was proposed that these $CD8^+$ T cells were non-classically MHC class I restricted but these findings are consistent with this newly described link between phagocytosis and MHC class I pathway and therefore these $CD8^+$ T cells may actually be classically restricted (Lewinsohn, Alderson et al. 1998; Canaday, Ziebold et al. 1999).

1.7.3.2 Non-cytosolic pathways

An alternative non-cytosolic pathway that leads to the presentation of antigens on MHC class I molecules has been proposed in which exogenous antigens are loaded onto MHC class I molecules through a vesicular recycling pathway (Pfeifer, Wick et al. 1993; Jondal, Schirmbeck et al. 1996; Song and Harding 1996; Chefalo and Harding 2001). In this model empty or unstable MHC class I molecules intersect with the MHC class II processing pathway in the endosomal system. The MHC class I molecules can be loaded with peptides or exchanged with higher affinity peptides derived from the lysosomal degradation of mycobacterial antigens (Sugita and Brenner 1995; Schirmbeck and Reimann 1996).

Uptake of exosomes containing mycobacterial lipids and proteins is another proposed pathway through which *M. tuberculosis* antigens could be presented on MHC class I molecules. It has been reported that BCG and *M. tuberculosis*-infected Mϕ contain and secrete vesicles containing mycobacterial lipids and proteins. These vesicles were present in the supernatant of cell cultures and were taken up by uninfected bystander cells, which may provide a means by which hydrophobic lipid antigens of *M. tuberculosis* can gain access to multiple processing pathways (Beatty, Rhoades et al. 2000; Beatty, Ullrich et al. 2001; Neyrolles, Gould et al. 2001).

Apoptosis of *M. tuberculosis*-infected Mϕ is likely to occur frequently during infection as most subsets of *M. tuberculosis*-reactive T cells and NK cells have been shown to induce apoptosis of infected Mϕ in addition to the induction of apoptosis by *M. tuberculosis* itself. Apoptosis of infected Mϕ results in the formation of apoptotic blebs containing mycobacteria. Uptake of these apoptotic blebs by non-infected DC or Mϕ can lead to the cross-presentation of mycobacterial antigens (Bellone, Iezzi et al. 1997; Rodriguez, Regnault et al. 1999; Schaible, Winau et al. 2003).

The relevance of any one of the pathways in the processing and presentation of *M. tuberculosis* antigens on MHC class I molecules *in vivo* is unknown. It is most likely that a combination of these occur in the priming and activation of *M. tuberculosis*-specific $CD8^+$ T cells. It is possible that that nature of mycobacterial antigen may be determine the pathways involved as secreted antigens may processed via cytosolic mechanisms whereas non-secreted antigens such as cell wall components may be cross-presented through uptake of exosomes or apoptotic bodies by uninfected APC.

1.7.4 Role of B cells

The role of B cells in the control of mycobacterial infection is unclear as mice that lack mature B cells do not display an increased susceptibility to infection. However, B cells are recruited to the lungs during *M. tuberculosis* infection in mice and form part of the TB granuloma. Moreover B cell deficient mice generate granulomas that contain a reduced number of neutrophils, macrophages and $CD8^+$ T cells (Johnson, Cooper et al. 1997; Bosio, Gardner et al. 2000). Thus, B cells may function in TB to enhance the recruitment of immune cells into the lungs through the production of chemokines.
In both human and bovine TB, antibody responses against mycobacterial antigens are associated with severe disease, suggesting that the development of a humoral immune response may reflect an inability of the hosts' immune system to control infection (Ritacco V et al 1991;Lightbody KA et al 2000) (Lightbody, Skuce et al. 1998). In support of this, patients with active TB show increased levels of IL-4 compared to healthy PPD-positive controls. Furthermore tuberculin skin testing of *M. bovis*-infected cattle significantly boosted greater antibody responses in non-vaccinated animals with severe disease compared to protected BCG-vaccinated animals (Lyashchenko, Whelan et al. 2004).

1.8 Role of the Granuloma in Tuberculosis

The granuloma is the hallmark of a protective immune response against mycobacterial infection. Granulomas function to contain the mycobacteria preventing dissemination and also limit tissue damage through shielding the surrounding tissue from the chronic inflammatory centre (Saunders, Frank et al. 1999). Inside the granuloma it is thought

that replication of the mycobacteria is controlled by the host anti-mycobacterial effector mechanisms. Reactivation of disease can occur when this immune system of the host becomes depressed and the balances tips in favour of the mycobacteria.

The typical characteristic of a TB granuloma in both humans and cattle but not in mice is the caseasous core comprised of necrotic cells. Tuberculous granulomas consist of epithelioid and monocyte-derived macrophages, DC, T cells, B cells, neutrophils and fibroblasts (Randhawa 1990; Peters and Ernst 2003; Co, Hogan et al. 2004; Johnson, Gough et al. 2006). Multinucleated Langhans giant cells are typically found in the centre of the granuloma surrounded by Mϕ and lymphocytes. In mice approximately two-fold more $CD4^+$ T cells than $CD8^+$ T cells accumulate at the site of *M. tuberculosis* infection (Mogues, Goodrich et al. 2001).

In the course of infection, mycobacteria are phagocytosed by resident Mϕ and DC which leads to the activation of these cells. Activated infected Mϕ form the core of the granuloma and produce a number of cytokines and chemokines which attracts other immune cells such as monocytes and neutrophils. Infected DCs migrate to the draining lymph and activate mycobacterial reactive $CD4^+$ and $CD8^+$ T cells which in turn traffic to the site of infection. The $CD4^+$ T cells and $CD8^+$ T cells function to kill infected cells and produce pro-inflammatory cytokines to enhance the killing of the mycobacteria by the infected cells. If the activation of the adaptive immune response controls mycobacterial replication than the mycobacteria enter a non-replicating dormant phase and the infection enters the latent stage (Wayne and Sohaskey 2001). At this stage fibroblasts form a wall around the lesion separating it from the surrounding tissue preventing bacterial dissemination. The centre of the granuloma becomes necrotic and it is thought that the mycobacteria contained in the centre of the granuloma are non-replicating (Wayne and Sohaskey 2001; Young 2002).

Thus, granulomas either provide a safe environment for mycobacteria to persist or alternatively they provide a hostile environment shielding the mycobacteria away from nutrients required for the replication. In patients with active TB a decrease in the numbers of $CD4^+$ and $CD8^+$ T cells was observed in the lumen of lung lesions, suggesting that *M. tuberculosis*-infected Mϕ present in these areas are not accessed by

M. tuberculosis-specific T cells (Kaplan, Post et al. 2003). Therefore in these TB patients the microenvironment of the lumen of the granuloma is permissive to *M. tuberculosis* survival.

The generation and maintenance of granulomas is essential for the control of *M. tuberculosis* and *M. bovis* infection and involves the interplay of many host factors. These include the production of cytokines such as TNF-α and IL-1β, expression of chemokines (MCP-1) and chemokine receptors (CCR2) whose main function is the trafficking of cells to site of infection (Flynn, Goldstein et al. 1995; Kaneko, Yamada et al. 1999; Peters, Scott et al. 2001).

1.9 Immune modulation by pathogenic mycobacteria

M. tuberculosis is one of the most successful persistent pathogens of man. Key to this success is the ability of the mycobacteria to modulate and evade the hosts' immune response. The main evasion mechanisms employed by mycobacterial involve the prevention of phagosomal fusion with microbicidal lysosome, resistance to toxic RNI and possible ROI, inhibition of activation and antigen presentation by infected Mϕ.

One of the first stages of infection is the phagocytosis of mycobacteria by alveolar macrophages. Phagocytosed particles follow a maturation process during which the phagosome fuses with microbicidal lysosomes which are acidic vesicles containing hydrolytic enzymes. Phagolysosomes are characterised by expression of lysosome-associated membrane glycoprotein 1 (LAMP1). Mycobacteria-containing phagosomes do not express LAMP1 and are only slightly acidic. Mycobacteria prevent the maturation of phagosomes, thereby avoids destruction by acidification and lysosomal enzymes. Furthermore mycobacteria maintain the environment of the phagosome by excluding vacuolar H^+-ATPases further avoiding acidification of the phagosome (Sturgill-Koszycki, Schlesinger et al. 1994; Xu, Cooper et al. 1994). Inhibition of phagosomal maturation by the mycobacteria has been proposed to be mediated by modulation of the phagosomal membrane.

This may include interference with the machinery that regulates fusion of the phagosomes with lysosomes. Small GTP-ases of the Rab family regulate the fusogenicity of phagosomes and mycobacteria-containing phagosomes were found to retain the Rab 5 protein. The Rab 5 protein is expressed on early endosomes and dissociates as the endosomes gain Rab 7 (Fratti, Backer et al. 2001). This retention of Rab5 also indicates the phagosomes containing mycobacteria are able to fuse with other vesicles in the early endosomal compartment and may provide a means by the mycoabcteria access the molecules required for its survival. SNARE proteins (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) are also involved in the fusion of endosomes. Mycobacteria-containing phagosomes have been reported to contain an aberrant form of the SNARE protein cellubrevin involved in phagosomal maturation (Fratti, Chua et al. 2002). Furthermore the mycobacterial cell wall component ManLAM was found to inhibit the recruitment of a tethering molecule EEA1 (early endosome autoantigen 1) which interacts with SNARE during endosomal fusion (Fratti, Backer et al. 2001).

The retention of TACO (tryptophan aspartate rich coat protein) on phagosome containing live mycobacteria was suggested to prevent fusion with lysosomes as transfection of TACO into TACO-deficient melanoma cell line enhances phagolysosomal fusion and an increase of mycobacterial killing (Schuller, Neefjes et al. 2001).

These findings indicate that selective inhibition of phagosomal fusion by the mycobacteria is likely to prevent the maturation of the phagosome and promote the survival of the mycobacteria.

Another mechanism by which mycobacteria are able to persist inside Mϕ is the resistance to killing by NO and RNI. *M. tuberculosis* has been shown to form an antioxidant complex which may catabolise and detoxify the RNI (Bryk, Lima et al. 2002). Moreover *M. tuberculosis* genes noxR1 and nox R2 can confer resistence to RNI in less virulent strains of mycobacteria (Ehrt S et al 1997; Ruan J et al 1999). Recently, the mycoabcterial proteasome was shown to protect *M. tuberculosis* against the microbicidal effects of NO (Darwin, Ehrt et al. 2003; Pieters and Ploegh 2003).

$CD4^+$ T cells recognise antigens presented on MHC class II molecules and are required for the control of mycobacterial infections. *M. tuberculosis* has been shown to inhibit antigen presentation by infected Mϕ. IFN-γ activation of Mϕ results in an up-regulation of MHC class II expression but the effect is absent in *M. tuberculosis* infected Mϕ (Hmama, Gabathuler et al. 1998). Furthermore *M. tuberculosis*-infected monocytes showed a reduced capacity to present tetanus toxoid compared to uninfected Mϕ (Gercken, Pryjma et al. 1994). This inhibition of MHC class II pathway has been proposed to be due to the intracellular sequestration of MHC class II molecules and a decreased expression of the class II transactivator (CIITA) (Hmama, Gabathuler et al. 1998; Wojciechowski, DeSanctis et al. 1999). This has also be attributed to decreased sensitivity to IFN-γ in *M. tuberculosis*-infected Mϕ. Refractoriness to IFN-γ in *M. tuberculosis*-infected Mϕ was mediated by the blocking a part of the IFN-γ signalling pathway by the mycobacteria (Ting, Kim et al. 1999). The 19 kDa mycobacterial lipoprotein binds TLR2 and has been shown to inhibited of IFN-γ mediated up-regulation of MHC class II expression in both human and mouse cells (Noss, Pai et al. 2001; Pecora, Gehring et al. 2006). A further immunomodulatory role for this lipoprotein was proposed in the induction of apoptosis in the infected Mϕ through binding to TLR (Lopez, Sly et al. 2003)

Recognition of *M. tuberculosis* is likely to occur through a number of TLRs and activation of these leads to the initiation of MAPK activity. Activation of these kinases can result in the synthesis of inflammatory mediaters and microbicidal molecules in Mϕ. Mycobacterial ManLAM has been shown to inhibit the activation of MAPK in human monocytes, indicating that *M. tuberculosis* is able to regulate the function of the host cell and protect itself against effectors functions of the Mϕ (Knutson, Hmama et al. 1998; Cobb 1999).

M. tuberculosis and *M. bovis* may also modulate the immune response by inducing the production of inhibitory cytokines by the infected Mϕ such as IL-10 and TGF-β (Hirsch, Hussain et al. 1996). These cytokines have been shown to be produced in excess by patients with active TB. This induction of these anti-inflammatory cytokines

is beneficial to the mycobacteria as they inhibit the effects of pro-inflammatory cytokines and directly inhibit T cell function.

Virulent mycobacteria such as *M. tuberculosis* and *M. bovis* have evolved a number of mechanisms to modulate the host immune response in order to promote survival and persistence. A clearer understanding of the mycobacterial products and the pathways involved in this regulation may provide potential targets for preventative and therapeutic agents against TB.

1.9 Memory Immune Responses

1.9.1 Generation and maintenance of memory $CD8^+$ T cells

Antigen specificity and memory are two defining features of the adaptive immune response that constitute its ability to specifically combat infection. The generation of protective long-term immunological memory forms the basis of an effective vaccine. It has been shown that some populations of memory T cells can be maintained for the lifetime of the host, up to 2 years in mice and 35-50 years in humans (Lau, Jamieson et al. 1994; Demkowicz, Littaua et al. 1996).

The known properties that endow memory cells to mediate recall responses and confer long-term protective immunity are attributed to an increased number of Ag-specific T cells, more rapid responses, migrate into peripheral tissues (near the site of microbial entry) and their ability to persist. Such properties are acquired during the differentiation of $CD8^+$ T cells from naïve to memory status, reflected by changes in their gene-expression profile resulting from rearrangement of chromatin structure and pattern of active transcription factors (Agarwal and Rao 1998). Furthermore some memory $CD8^+$ T cells constituitively express messenger RNA transcripts for IFN-γ and cytotoxic molecules such as perforin and granzyme B, proteins that are not commonly expressed in naïve $CD8^+$ T cells. (Bachmann, Barner et al. 1999); (Veiga-Fernandes, Walter et al. 2000). Memory T cells express a different pattern of cell surface molecules involved in cell adhesion and chemotaxis compared to naïve $CD8^+$ T cells, allowing memory cells to survey the periphery for antigen.

Ahmed and GrAy described three stages of a T cell response as it differentiates into a memory cell. Firstly, the expansion phase, during which naïve $CD8^+$ T cells in the

lymphoid tissues encounter antigen (Ag), clonally expand and differentiate into effector $CD8^+$ T cells (Ahmed and Gray 1996). Optimal activation of $CD8^+$ T cells has been proposed by Mescher et al to require 3 signals these are antigen recognition, co-stimulation and presence of cytokines either IL-12 or IFN-α (Mescher, Curtsinger et al. 2006). The cytotoxic T lymphocytes (CTLs) generated from the naïve $CD8^+$ T cells rapidly eliminate infectious pathogens through a combination of effector functions that include secretion of cytokines IFN-γ and TNF-α and cytotoxic molecules such as perforin and granzymes. The contraction phase follows the clearance of the infection during which the majority of effector T cells (90%) undergo activation induced cell death (AICD). It is thought that a small population of effector T cells survive AICD and enter the memory phase in which the number of Ag-specific T cells stabilises and is maintained. The mechanisms that promote the survival of a small fraction of the effectors cells generated during an infection are not fully understood. Recent reports using a mouse model of acute viral infection and *Listeria monocytogenes* demonstrated that sustained expression of the IL-7 receptor identified effector $CD8^+$ T cells that survived AICD and became memory cells (Kaech, Tan et al. 2003; Badovinac, Porter et al. 2004) Expression of IL-7R on effector $CD8^+$ T cells correlated with increased expression of anti-apoptotic molecule Bcl-2. These findings suggest that active receptor signaling is required for the survival of $CD8^+$ T cell subsets (Kaech, Tan et al. 2003).

The model described by Ahmed and Gray does not take into account the differentiation of memory T cells during chronic infection in which antigen is not eliminated. Some of the worlds most successful pathogens employ strategies to avoid detection and elimination by the hosts immune response such as *M. tuberculosis*, HIV and hepatitis C virus. It is clear that memory T cells that are generated during chronic viral infections are defective in their ability to exhibit effector functions which may be due to repeated stimulation leaving the memory cells "exhausted". A recent study showed that expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death receptor (PD-1) was observed in exhausted memory T cells but not long-term functioning memory cells (Barber, Wherry et al. 2006). It is thought that engagement of these receptors leads to an inhibitory signal being delivered to the memory T cells.

Chronic infections have also been shown to prematurely age the memory T cells induced by the infection. The immune system in older subjects has a decreased capacity to respond to new infections or to contain persistent infections. This defect has been

attributed to changes in the composition of the T cell compartment with an increased number of primed/memory $CD8^+$ T cells and decreased number of naïve $CD8^+$ T cells (Fagnoni, Vescovini et al. 2000). Furthermore $CD8^+$ T cells from elderly subjects display a reduced expression of molecules involved in co-stimulation CD28 and CD27. Expression of CD27 and CD28 are lost after repeated rounds of antigen stimulation and a high number of cell divisions (Hendriks, Gravestein et al. 2000; Brzezinska, Magalska et al. 2004). Therefore in elderly subjects there is an accumulation of highly differentiated $CD8^+$ T cells that have a reduced ability to proliferate in response to antigen, this occurence has been termed replicative senescence. Replicative senescence describes the increased telomere erosion and a loss of proliferative capacity of memory T cells upon long-term antigenic exposure.

It has been suggested that replicative senescene may occur in young individuals who have chronic infections. Similar changes in memory $CD8^+$ T cells have been observed during chronic viral infections as in aged subjects shown by high proportions of $CD45RA^+CD27^-CD28^-$ T cells correlates with Cytomeglovirus-seropositivity (CMV) and in HIV-infected individuals the HIV-specific $CD8^+$ T cells lack CD28 and CD27 (Dalod, Sinet et al. 1999; Tomiyama, Oka et al. 2000; Appay, Dunbar et al. 2002; Kalayjian, Landay et al. 2003; Kuijpers, Vossen et al. 2003). Moreover $CD8^+CD28^-$ T cells from HIV-infected individuals have identical telomere length to that of centenarians and an increased number of $CD28^-$ T cells correlates with disease progression (Effros, Boucher et al. 1994; Effros, Allsopp et al. 1996; Lewis, Yang et al. 1999; Gamberg, Pardoe et al. 2004).

In order to fully understand the pathways that lead to the generation of memory cells it is important to look at the models of $CD8^+$ T cell differentiation that have been proposed. It was initially thought that $CD8^+$ T cell differentiation was dependent upon repeated encounters with Ag. In this model each daughter cell must be stimulated with Ag and differentiation would halt upon Ag removal. In contrast to this theory Kaech and Ahmed demonstrated that $CD8^+$ T cells were developmentally programmed to clonally expand (divide at least 7-10 times) and differentiate into CTL's after only a brief encounter with Ag. They found that the same duration of antigenic stimulation (2-24h) that drove naïve $CD8^+$ T cell to proliferate was also sufficient for them to commit to differentiate into effector cells that secrete IFN-γ, TNF-α and display cytotoxicity.

Further Ag stimulation was not required for completion of the developmental programme, thus suggesting that commitment to clonal expansion and effector T cell differentiation is tightly coupled (Kaech and Ahmed 2001).

In addition, Lanzavecchia et al have proposed the progressive differentiation model in which only the fittest cells are selected to become memory T cells. Fitness is determined by the level of stimulation received by the differentiating $CD8^+$ T cells and only cells that receive optimal level of stimulation that also access and utilise survival signals differentiate into memory cells (Lanzavecchia and Sallusto 2002). In contrast a divergent model of memory T cell generation has been suggested in which newly activated naïve T cells can directly differentiate into memory T cells without becoming effector cells (Manjunath, Shankar et al. 2001).

The development and maintenance of memory $CD8^+$T cells is key to providing protection against infection and disease. Memory T cell populations are maintained in the steady state for long periods of time by slow continuous proliferation and it is thought that rate of homeostatic division equals the rate of cell death as the number of memory $CD8^+$T cells remains relatively constant (Tuma and Pamer 2002). Maintenance of memory $CD8^+$ T cells has been shown to be independent of antigen and MHC class I molecules (Zajac, Blattman et al. 1998). The involvement of Ag-independent factors such as cytokines has been demonstrated in transgenic mice that constitutively express IL-15, as these mice generate higher numbers of memory $CD8^+$ T cells with an increased expression of anti-apoptotic protein Bcl-2 (Yajima, Nishimura et al. 2002). In addition, IL-15 and IL-15Rα deficient mice display a selective reduction in the proportion of memory $CD8^+$ T cells (Lodolce, Boone et al. 1998; Kennedy, Glaccum et al. 2000). Recently, IL-15 has been shown to preferentially stimulate the proliferation of murine $CD8^+$ T cells that display the memory phenotype $CD44^{high}$. Furthermore, culturing with IL-15 was shown to promote the survival of both memory ($CD44^{high}$) and naïve ($CD44^{low}$) T cells via up-regulation of $Bcl\text{-}_2$ levels in both cell types, and an elevated expression of $Bcl\text{-}_{XL}$ in $CD44^{high}$ $CD8^+$ T cells (Berard, Brandt et al. 2003).

Studies in humans have proposed a role for IL-15 and IL-7 in the homeostasis of $CD8^+$ T cells. IL-15 has been shown to inhibit T cell apoptosis and the alpha chain of the IL-15 receptor is up-regulated upon activated $CD8^+$ T cells (Chae, Nosaka et al. 1996;

Bulfone-Paus, Ungureanu et al. 1997). More recently, it has been suggested that memory/effector $CD8^+$ T cells are more responsive to IL-15 compared to naïve T cells as the addition of exogenous IL-15 preferentially induced proliferation and cytokine production of anti-CD3 activated $CD8^+$ T cells that expressed the memory cell phenotypes $CD45RA^-CD62L^-$ and $CD45RA^+CD62L^-$ in both healthy and HIV-infected individuals (Mueller, Bojczuk et al. 2003). In addition to its activating properties, IL-15 was also shown to inhibit FAS (CD95) induced apoptosis of both human $CD4^+$ and $CD8^+$ T cells (Mueller, Makar et al. 2003).

Thus, IL-15 is proposed to be important in the survival and maintenance of murine memory and naïve $CD8^+$ T cells *in vivo* and a potent activator and survival factor of human and murine memory $CD8^+$ T cells *in vitro* (Kennedy, Glaccum et al. 2000; Marks-Konczalik, Dubois et al. 2000; Berard, Brandt et al. 2003).

1.9.2 Definition of Memory $CD8^+$ T cell subsets

Attempts have been made to identify memory $CD8^+$ T cell subsets based upon their immediate effector function and expression of cell surface molecules involved in cell adhesion and migration. Initial studies in humans demonstrated that differentially spliced isoforms of the leukocyte common antigen CD45 could be used to discriminate between populations of naïve and memory cells (Akbar, Terry et al. 1988). CD45RA is the highest molecular weight (MW) isoform and was shown to be expressed by a population of cells that divide approximately once every two years but when activated by specific antigen they lose expression of CD45RA and express the low MW isoform CD45RO (Michie, McLean et al. 1992). The $CD45RO^+$ population of T cells responds rapidly to recall antigens and divide once every 2 weeks in vivo(Michie, McLean et al. 1992).

However, this differentiation is complicated by the findings of recently studies using MHC class I tetramers incorporating Epstein Barr Virus (EBV) and Human Cytomegalovirus (HCMV) peptides, which demonstrated the presence of Ag-specific T cells within the $CD45RA^+$ and $CD45RO^+$ populations. Thus suggesting that a subset of memory $CD8^+$ T cells exist in the stable CD45RA state during chronic infection (Callan, Annels et al. 1998; Gillespie, Wills et al. 2000). Further evidence suggests that a significant number of herpes virus specific $CD8^+$ T cells revert from $CD45RO^+$ to $CD45RA^+$ after priming (Faint, Annels et al. 2001). Phenotypic characterisation of

these CD45RA$^+$ T cells showed that they are CD11a^{hi} CCR7^{lo} CCR5$^+$ CD28$^-$ and heterogeneous in their expression of L-selectin (CD62L) (Wills, Okecha et al. 2002). These cells were also shown to be functional, as upon stimulation they are potent producers of IFN-γ and express high levels of perforin (Faint, Annels et al. 2001). CD45RA$^+$ memory cells preferentially migrate into tissues and were under-represented in the lymph nodes compared with levels of CD45RO$^+$ memory CD8$^+$ T cells. It was suggested that this population of memory cells came from highly differentiated CD8$^+$ T cells as they had short telomeres similar to that of CD45RO$^+$ cells and expressed high levels of anti-apoptotic markers Bcl-2 and Bcl-x_L (Champagne, Ogg et al. 2001; Fearon, Manders et al. 2001). It subsequently reported that these cells may not be terminally differentiated as they divide and proliferate when activated together with antigen and co-stimulation which maybe provided by IL-2, CD4$^+$ T cell help, IL-15 or IL-21 (van Leeuwen, Gamadia et al. 2002).

It is unknown whether these CD45RA$^+$ CD8$^+$ T cells arise by a distinct pattern of differentiation from CD45RA$^+$ naïve T cells that never express CD45RO or are generated from reversion of CD45RO$^+$ CCR7$^+$ central memory cells upon re-stimulation.

Expression of the tumour necrosis factor receptor (TNFR) family member CD27 and chemokine receptor CCR7 have been proposed to distinguish between CD45RA$^+$ naïve and CD45RA$^+$ Ag-experienced cells with naïve T cells being CD27$^+$CCR7$^+$ and terminally differentiated T cells being CD27$^-$ CCR7$^-$ (Hamann, Baars et al. 1997). But recently, Wills et al argued that CD27 expression does not divide naïve T cells and RA primed cells as they showed that the presence of a population of HCMV specific CD8$^+$ T cells expressing the phenotype CD45RA$^+$CD28$^-$CCR7$^-$CD27$^+$ (Wills, Okecha et al. 2002).

Sallusto et al described two populations of memory CD8$^+$ T cells that are distinct in their migratory capacity (anatomical location) and effector functions and can be identified by their expression of CD45RO, CCR7 and CD62L (Sallusto, Lenig et al. 1999). CCR7 is a chemokine receptor that binds CCL19 and CCL21 expressed by endothelial cells in the lymph nodes causing firm arrest and initiation of extravasation (Campbell, Bowman et al. 1998). CD62L interacts with peripheral-node addressin (PNAd) present on high endothelial venules (HEV) mediating attachment and rolling of cells to the endothelium (Arbones, Ord et al. 1994). A population of memory CD8$^+$ T

cells expressing the phenotype $CD62L^+$ $CCR7^+$ $CD45RA^-$ have been shown to migrate efficiently to the peripheral lymph nodes, these were termed Central memory T cells (Weninger, Crowley et al. 2001). It was demonstrated that this subset of memory $CD8^+$ T cells lack immediate effector function but upon Ag-stimulation lose CCR7 expression and gain effector functions.

The other subset of CD8 memory T cells was identified by a lack of CD62L and CCR7 expression and exhibited immediate effector function, these were termed effector memory T cells (Sallusto, Lenig et al. 1999). These cells preferentially migrate into the peripheral tissues such as the liver and lungs (Manjunath, Shankar et al. 2001). It is thought that two types of effector memory $CD8^+$ T cells exists and can be identified as being either $CD45RA^-CD62L^-CCR7^-$ or $CD45RA^+CD62L^-CCR7^-$ (Sallusto F et al 1999; Champagne et al 2001).

Thus, in summary two models of memory $CD8^+$ T cells is humans have been proposed the first being based upon the expression of co-stimulatory molecules CD28 and CD27 together with CD45RA to define three subsets being naïve ($CD45RA^+CD27^+CD28^+$), effector ($CD45RA^+CD27^-CD28^-$) and memory cells ($CD45RA^-CD27^+CD28^+$ and $CD45RA^+CD27^-CD28^+$). This model is limited as it is based upon results taken from chronic viral infections, during which the antigen persists.

The second model involves a population of peripheral tissue homing effector memory T cells capable of immediate effector functions ($CD45RA^-CD62L^-CCR7^-$ or $CD45RA^+CD62L^-CCR7^-$) and could target invading pathogens at the site of entry. Also described in this model are central memory $CD8^+$ T cells which lack immediate effector function, recirculate preferentially through the lymph node and function to proliferate and generate a second wave of antigen specific effector T cells ($CD62L^+$ $CCR7^+$ $CD45RA^-$).

At present little is known regarding $CD8^+$ T cell memory subsets in cattle, although it has been shown that after *Theileria parva* infection, CTL precusors exist in $CD8^+CD45RO^+$, $CD8^+CD45RO^-$ and $CD8^+$ $CD45RB^+$, $CD8^+CD45RB^-$ T cell subsets at a similar frequency (Howard, Sopp et al. 1991); (Bembridge, MacHugh et al. 1995).

CHAPTER TWO: AIMS AND OBJECTIVES

The increasing incidence of *M. bovis* infection in UK cattle herds demonstrates a failure in the current control strategy, the test and slaughter policy. There is a need for improved control measures that should include an effective vaccine and a more proficient diagnostic test. To achieve either of these a greater understanding of the immune responses to infection with *M. bovis* in cattle is required.

Studies in mouse models of TB have shown that $CD8^+$ T cells are required for protection against *M. tuberculosis* infection. *In vitro* studies in humans have shown that $CD8^+$ T cells are induced by BCG vaccination and *M. tuberculosis* infection. In cattle, BCG vaccination confers a degree of protection against *M. bovis* challenge and both $CD4^+$and $CD8^+$ cells have been shown to be involved. The nature of $CD8^+$ T cell responses in infection with mycobacteria is poorly characterised.

Thereby, the aim of this study is to further the current knowledge of memory $CD8^+$ T cell responses to mycobacteria in cattle. To achieve this aim, a number of objectives were established: (1) to phenotypically characterise memory and naïve $CD8^+$ T cells in cattle, (2) to investigate whether BCG-vaccination induces a memory $CD8^+$ T cell response and (3) to determine the role these cells play in response to *M. bovis* infection.

1. Characterise naïve and memory $CD8^+$T cells using surface phenotype and expression of effector molecules.
    a. Analyse the expression of cell-surface and effector molecules by $CD8^+$T cells in blood from animals of different ages
    b. Compare the expression of cell-surface and effector molecules by $CD8^+$T cells present in peripheral tissue and lymph nodes.
2. Investigate whether BCG-vaccination of cattle induces memory $CD8^+$T cells
    a. Establish and optimise an *in vitro* assay that is consist and reliable for the detection of mycobacterial reactive $CD8^+$T cells.
    b. Phenotypically and functionally define the responding $CD8^+$T cells and investigate mechanisms by which they could promote the clearance of mycobacterial infection
    c. Determine whether the memory $CD8^+$T cells induced by BCG vaccination mediate recall responses *in vivo*.

3. Investigate whether *M. bovis* infection of cattle induces a $CD8^+$ T cell response
    a. Identify whether changes in the proportion of circulating $CD8^+$ T cell subsets are induced by *M. bovis* infection
    b. Determine whether mycobacterial reactive $CD8^+$ T cells are generated after infection of non-vaccinated and BCG- vaccinated cattle with *M. bovis*.
    c. Compare the $CD8^+$ T cell response induced by *M. bovis* infection of BCG vaccinated and non-vaccinated animals
    d. Investigate whether mycobacterial reactive $CD8^+$ T cells contribute to the immune response in the lungs of infected animals, one of the primary sites of infection.

This study will provide a model to investigate the development of $CD8^+$ T cell responses induced by mycobacteria and may be used in the evaluation of new TB vaccination strategies. The results of this study may lead to the development of more effective vaccination strategies to target and amplify subsets of memory $CD8^+$ T cells with specific migratory and effector functions which may enhance protection against *M. bovis*.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Media Formulation

3.1.1 Media for culture of mycobacteria

All media used in bacteriology work was kindly prepared by the Media Department at the Institute for Animal Health and obtained from Difco Ltd unless otherwise stated.

Middlebrook 7H9 broth

Middlebrook broth base (4.7g dissolved in 900ml H_20)	
OADC supplement	10% (v/v)
Tween-80	0.05%
Sodium pyruvate	0.4% (w/v)

Middlebrook 7H10 Agar base

Middlebrook 7H10 base (19g dissolved in 900ml H_20)	
OADC supplement	10% (v/v)
Sodium pyruvate	0.4% (w/v)
Pancreatic digest casein	1 g

Modified 7H11 Agar base

Middlebrook 7H10 base (19g dissolved in 900ml H_20)	
OADC supplement	10% (v/v)
Sodium pyruvate	0.4% (w/v)
Pancreatic digest casein	1 g

3.1.2 Tissue culture media

General cell culture media (TCM)

RPMI 1640 with Glutamax I and HEPES (Invitrogen)	90 ml
Foetal calf serum (IAH-media)	10 ml
2-Mercaptoethanol (2-ME) (Sigma)	5×10^{-5} M
Gentamycin (Sigma)	50 μg ml^{-1}

Dendritic cell differentiation media

RPMI 1640 with Glutamax I and 25mM HEPES	90 ml
Heat-inactivated Foetal calf serum	10 ml
2-Mercaptoethanol (2-ME)	5×10^{-5} M
Gentamycin	50 μg ml^{-1}
Rbo IL-4	200 U ml^{-1}
Rbo GM-CSF	0.2 U ml^{-1}

(Units based on induction of half-maximal proliferation in bone marrow precursor cells)

Macrophage differentiation media

Iscoves Modified Dulbeccos Medium (DMEM) with GLUTAMAX and 25mM HEPES (Gibco Ltd, Paisley)	90 mls
Heat-inactivated Foetal calf serum	10mls
2-Mercaptoethanol (2-ME)	5×10^{-5} M
Gentamycin	50 μg ml^{-1}
Rbo GM-CSF	0.2 U ml^{-1}

(Units based on induction of half-maximal proliferation in bone marrow precursor cells)

3.1.3 General buffers and solutions

All buffers used in cell culture were filtered sterilised using a 0.22 μm filter (Sterillin)

Phosphate buffered saline (PBSa)

Sodium chloride	170 mM
Potassium chloride	3.4 mM
Sodium phosphate	9.2 mM
Potassium phosphate	1.8 mM

pH 6.8

0.1% Trypan Blue solution

0.4% Trypan blue solution in PBSa (IAH-media services)

FACS washing buffer

PBSa + 1% Bovine serum albumin + 0.1 % Sodium Azide

Fixative buffer

1% Paraformaldehyde in PBSa (IAH-media services)

FACSFlow buffer

1% Bovine serum albumin (Sigma) in FACSFlow (BD Biosciences)

Sorting buffer

1% Bovine serum albumin in PBSa (IAH-media services)

3.1.4 Gel Buffers

TBE buffer

Tri	1M
Boric acid	0.9M
EDTA	0.01M

Gel loading buffer

Bromophenol blue	0.25 % (w/v)
Xylene cyanol	0.25 % (w/v)
Gylcerol	30 % (w/v)
Tris acetate	40 mM
EDTA	1 mM

4M GTC solution

All components were obtained from Sigma and were certified Rnase-free

4M Guanidine thiocyanate

0.5% sodium N-lauryl sarcosine

25mM Tri-sodium citrate

0.5% Tween-80

Dissolved in RNase-free H_20

0.1M of 2-ME was added prior to use

3.1.5 ELISA buffers and substrates

Coating buffer

Carbonate/Bicarbonate (Sigma-Aldrich)	1 capsule
Super Q water (SQW) (IAH-media services)	100 ml

pH 9.6

Blocking buffer

1 mg/ml Sodium casein (Sigma) in PBSa (IAH-media services)

Washing buffer

0.05% Tween 20 in PBSa (IAH-media services)

Reagent Diluent

0.05% Tween 20 + 1mg/ml Sodium casein + PBS (IAH-media services)

Sodium Acetate solution

0.1 M Sodium acetate (anhydrous) in Super Q water (IAH-media services)

pH adjusted using pH meter by adding 0.1M citric acid (BDH) to pH 6.0

TMB solution 10mg/ml

10 mg/ml TMB in DMSO

Substrate solution (per 10 mls)

Sodium acetate solution	9.9 mls
TMB solution	100 µl
Hydrogen peroxide solution (30%)	1.5 µl

3.2 Antibody Tables

3.2.1 Monoclonal Antibodies

Antibody /clone	Antigen recognised	Isotype	Species	Reference	Supplier
CC32	CD62L	IgG1	Mouse anti-bovine	(Howard, Sopp et al. 1992)	IAH
CC76	CD45RA	IgG1	Mouse anti-bovine	(Bembridge, Howard et al. 1993)	IAH
IL-A116	CD45RA	IgG3	Mouse anti-bovine	(Bembridge, Howard et al. 1993)	IAH
IL-A111	CD25	IgG1	Mouse anti-bovine	(Naessens, Sileghem et al. 1992)	IAH
CC62	CD26	IgG2b	Mouse anti-bovine	(Sopp, Howard et al. 1993)	IAH
GB21A	TCR1	IgG2b	Mouse anti-bovine	(Wyatt, Madruga et al. 1994; MacHugh, Mburu et al. 1997)	Bill Davis, Washingston State University
CC30	CD4	IgG1	Mouse anti-bovine	(Bensaid and Hadam 1991)	IAH
CC39	WC1	IgG1	Mouse anti-bovine	(MacHugh, Mburu et al. 1997)	IAH
CCG33	CD14	IgG1	Mouse anti-human	(Sopp, Kwong et al. 1996)	IAH
CC42	CD2	IgG1	Mouse anti-bovine	(Davis and Splitter 1991)	IAH
	CD27	IgG1	Mouse anti-bovine		
F848 4E8	CD28	IgG1	Mouse anti-bovine	From Geraldine Taylor, IAH	IAH
MM1A	CD3	IgG1	Mouse anti-bovine	(MacHugh, Mburu et al. 1997)	Bill Davis, Washingston State University
CD8-APC	CC63-APC	IgG2a	Mouse anti-bovine	(MacHugh, Bensaid et al. 1991)	IAH
CC17	CD5	IgG1	Mouse anti-bovine	(Howard, Parsons et al. 1988)	IAH
CC302	IFN-γ	IgG1	Mouse anti-bovine	(Sopp and Howard 2001)	IAH
δG9	Perforin – FITC	IgG2b	Mouse anti-human		BD Pharmingen

3.2.2 Isotype Controls

Antibody	Antigen recognised	Species	Isotype	Supplier
AV20	Avian B-cells	mouse anti-chicken	IgG1	IAH
AV29	Avian control	mouse anti-chicken	IgG2b	IAH
AV37	Avian spleen cell subset	mouse anti-chicken	IgG2a	IAH
AV20-FITC	Avian control	mouse anti-chicken	IgG1-FITC	IAH
TRT1	Avian control	mouse anti-chicken	IgG1	IAH
TRT3	Avian control	mouse anti-chicken	IgG2a	IAH
IgG3		Mouse antibody	IgG3	Sigma-Aldrich, Saint Louis, USA.
IgG2b control	Murine	Mouse antibody	IgG2b-FITC	BD Pharmingen

3.2.3 Secondary Fluorochrome Conjugates

Conjugate	Specificity	Supplier
FITC	Goat anti-mouse IgG1	Southern Biotechnology
PE	Goat anti-mouse IgG1	Southern Biotechnology
Tricolour	Goat anti-mouse IgG1	Caltag Laboratories
Tricolour	Goat anti-mouse IgG2b	Caltag Laboratories
PE	Goat anti-mouse IgG2b	Southern Biotechnology
FITC	Goat anti-mouse IgG2b	Southern Biotechnology
PE	Goat anti-mouse IgG3	Southern Biotechnology
FITC	Goat anti-mouse IgG3	Southern Biotechnology
Alexa Fluor 633	Goat anti-mouse IgG1	Molecular Probes

3.3 Experimental animals

All experiments were performed under the regulations of the Home Office Scientific Procedures Act (1986). The animals used in these experiments were conventionally reared British Friesian Holstein calves (*Bos Taurus*) housed at the Institute for Animal Health. Animals were selected based upon age and vaccination/infection status. Animals were infected with 10^4 cfu of *M. bovis* administered either intranasally or intra-tracheally.
Animals used in this study were vaccinated with 10^6 cfu of BCG subcutaneously or intradermally with 10^9 plaque forming units (pfu) of VV-Ag85 (IAH).

3.4 Preparation of Mycobacterial stocks

3.4.1 Culture of *M. bovis*

The *M. bovis* strain AF 2122/97 was used throughout this study and was originally isolated in the UK in 1997 from a cow harbouring caseous lesions in the lung and bronchomediastinal lymph node. This strain has been fully sequenced (Garnier, Eiglmeier et al. 2003) and has been previously reported as being fully virulent in cattle (Villarreal-Ramos, McAulay et al. 2003). *M. bovis* cultures were prepared by gentle agitation in flasks of 10^6-10^7 mycobacteria in 10mls of modified Middlebrook 7H9 broth for 7 days at 37^0C and 100 rpm (GFL; model 3031). The cultures were then transferred into 90 ml of 7H9 media and cultured for a further 3 days at which point the culture was deemed to be in mid-exponential phase. Aliquots of the mycobacteria were stored at -80^0C.
All work with *M. bovis* was carried out under ACDP III conditions and work with *M. bovis*-BCG and *M. avium* was carried out under ACDP II conditions at the Institute for Animal Health.

3.4.2 Culture of *M. bovis* BCG

M. bovis-Bacillus Calmette Guerin (BCG), strain Pasteur, was used throughout this study and was obtained from Veterinary Laboratories Agency (VLA), Weybridge, Surrey. Cultures were prepared by incubating 10^6-10^7 mycobacteria in 10mls of Middlebrook 7H9

media (3.1 Media formulations) for one week at 37^0C. The mycobacteria was then transferred to a 500ml glass bottle and made up to a final volume of 100mls with 7H9 broth and cultured for a further two weeks at 37^0C. The bacterial culture was harvested and stored in 1ml aliquots at -70^0C.

3.4.3 Titre of frozen Mycobacteria

Enumeration of viable mycobacteria was performed by making ten-fold serial dilutions of the thawed stock in 7H9 broth. Dilutions were made in triplicate and plated out onto 7H10 agar plates for BCG and *M. avium* and onto 7H11 plates for *M. bovis* and incubated at 37^0C for 3 weeks. After this time the number of colonies present for each dilution was counted, from this the titre was calculated.

3.5 Cell Preparation Methods

3.5.1 Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected by jugular venupuncture and transferred into a sterile plastic 50ml Falcon tube containing 10U/ml of sodium heparin (Leo Laboratories Ltd.) to prevent coagulation. In a 50 ml Falcon polypropylene tube 15 ml of blood was diluted in an equal volume of filtered phosphate buffered saline (PBSa) and then underlayed with 15 ml of Histopaque 1083 (Sigma-Aldrich) to form a density gradient. The gradients were centrifuged at 1200 x *g* for 30-40 minutes (min) at room temperature with the centrifuge brake off during deceleration. The cells forming the interface layer between the histopaque and plasma were harvested and washed three times with PBS. The first wash was centrifuged at 660 x *g* for 10min with the second wash at 300 x *g* for 8 min and the final wash at 400 x *g* for 5min all at 4^0C with brake on. The cells were resuspended in appropriate media to determine cell count and viability

3.5.2 Preparation of Brochoalveolar Lavage cells (BAL)

Animals were killed using captive bolt method followed by exsanguinations. Lungs were removed with the trachea attached from animals at necropsy. Lungs were washed twice by pouring 2 litres (l) of PBS down through the trachea then the lung tissue was massaged well before the PBS was harvested into a sterile beaker. The recovered lung wash was transferred into large centrifuge buckets and the cells were pelleted at 300 x g for 15 min. The cells were resuspended in tissue culture media (TCM) (3.1 Media formulations) to determine cell count and viability. Alveolar macrophages represent approximately 90% of the total cells present in lung wash, thus BAL cells were incubated in tissue culture flasks at 10^7/ml in TCM (3.1 Media formulations) for 1 hour (hr) with the flask lying flat to allow the macrophages to adhere. Supernatants were removed and pelleted by centrifugation at 400 x *g* for 5 min. The pellet was resuspended in TCM to determine cell count and viability.

3.5.3 Preparation of single cell suspensions from lymph nodes

Lymph nodes were removed at necropsy. External fat and connective tissue was carefully removed and an incision was made down through the middle of the node to expose the interior of the node. The node was placed into a Petri dish containing PBSa and a scalpel was used to score the interior of the node and gently scrap the entire node to release cells into suspension. The collected cell suspension was pipetted through a 40 μm cell strainer (BD Bioscience), diluted in PBS and live cells were isolated using density centrifugation as described above. The cells were resuspended in appropriate media for determination of cell count and viability.

3.5.4 Cell Viability

Viable cells were counted using the trypan blue exclusion method. A small volume of cells was diluted with an equal volume of trypan blue solution. Only live cells, as determined by exclusion of stained cells, were counted on a haemocytometer viewed under a light

microscope (Leica). The proportion of stained cells never exceeded 10% of the total cell count. The number of cells present in the sample was calculated as follows:

Number of viable cells in 25 squares x Dilution factor x 10^4 = Number of cells / ml

3.6 Flow cytometry

3.6.1 Testing monoclonal antibodies to confirm specificity and optimise working dilutions

PBMC were resuspended in FACs washing buffer (3.1 Media formulations) and 10^6 cells were added per well to a 96-well round bottomed plate (Nalge, Nunc International). Serial dilutions of the primary antibody were made in FACS washing buffer and 25µl of each dilution was added to the PBMC and incubated for 10 mins at room temperature. Cells were then washed with 100µl of FACs washing buffer and centrifuged at 800 x *g* for 2 min. Supernatant was flicked off and the cells were washed for a second time. Cells were then incubated with 25µl of a fluorochrome conjugate for 10 min at room temperature. Cells were then washed as described above and analysed on the fluorescence-activated cell sorter (FACS). Fluorochrome conjugates were also tested in a similar manner in which serial dilutions were made and they were tested against a number of antibody isotypes to confirm specificity and test cross-reactivity with other isotypes.

3.6.2 Three and four colour flow cytometry

Cells to be used for flow cytometry were resuspended in FACs washing buffer (3.1 Media formulations) and incubated in 96 U-well Microplates (Nalge Nunc International) at 5 x10^5-10^6 per well with 25µl of antibody (3.2 Antibody tables) at pre-determined optimum concentrations for 10min at RT. Cells were then washed three times with FACs washing buffer and pelleted at 400 *g* for 2min at 4^0C. Unconjugated primary antibodies were then fluorescently labelled by incubating cells with 25µl of anti-mouse isotype specific Ig conjugated to a fluorochrome (3.2 Antibody tables) for a further 10min at RT. Cells were

then washed as before and resuspended in FACS fixative buffer (3.1 Media formulations) and stored at 4^0C.

3.6.3 Intracellular cytokine staining

Cells were incubated in TCM at 10^7/ml with phorbol 12-myristate 13-acetate (Sigma-Aldrich), ionomycin (Calbiocham-Novabiochem) and brefeldin A (Sigma-Aldrich) (PMA/I/BFA) (to give a final concentration of 50ng/ml, 1µg/ml and 10µg/ml respectively) or just BFA alone (10µg/ml) for 5 hr at 37^0C 5% CO_2. Cells were washed in FACS washing buffer before being stained for surface markers as described 2.3.5. Cells were incubated at room temperature for 10 min in fixative buffer and washed. The cells were then incubated at room temperature for 10 min in permeabilsation solution (Becton Dickinson, UK), washed and incubated with a pre-determined optimum concentration of intracellular cytokine antibody for 30 min, in the dark at room temperature. The cells were washed three times and resuspended in washing buffer and stored at 4^0C.

PMA has an analogous structure to Diacylglycerol (DAG) which is an allosteric activator of protein kinase C (PKC) thus can activate T-cells. PMA was used in conjunction with Ionomycin, a calcium ionophore, which extracts Ca^{2+} from aqueous to organic phase, raising intracellular calcium and thus increasing T-cell activation. BFA inhibits the transport of vesicles from the ER to the cell surface.

3.6.4 Intracellular perforin staining

Cells were washed in FACS washing buffer (3.1 Media formulations) before being stained for surface markers as described 2.3.5. Cells were incubated for 10 min in fixative buffer (3.1 Media formulations) and washed. The cells were then incubated for 10 min in Permeabilisation solution (Becton Dickinson, UK), washed and incubated with 25 µl of anti-human perforin antibody (BD Pharmingen) for 30 min, in the dark. The cells were washed three times and resuspended in washing buffer and stored at 4^0C.

3.6.5 CFDA staining

Carboxyfluorescein diacetate succinimidyl ester (CFDA) passively diffuses into the cell membrane. CFDA is non-fluorescent until its acetate groups are cleaved by intracellular esterases to yield the highly fluorescent amine-reactive carboxyfluorescein succinimidyl ester (CFSE). CFSE is retained by cell through cell division and results in the daughter cells having a slightly lower intensity of fluorescence compared to the original cell. This allows the tracking of the number of times a particular cell has divided.

PBMC were resuspended in warmed PBSa at a concentration of 10^7/ml and mixed with an equal volume of CFDA working solution (5μM) to give a final concentration of cells 5 x 10^6 cells/ml and CFDA 2.5μM. PBMC were incubated in a water bath at 37^0C for 15 min. PBMC were than pelleted by centrifugation (400 x *g* for 8 min) and resuspended in pre-warmed TCM and incubated in the water bath for a further 30 min. PBMC were pelleted as before and resuspended in TCM for culture.

3.6.6 Flow cytometry: Analysis and Gating

Samples were read on FACSCalibur (Becton Dickinson). Fifty to one hundred thousand events were collected and analysed using FCS Express (De Nova Software). The lymphocyte population was identified on the basis of forward and and orthoganol light scatter. The lymphocytes formed a defined population and were gated appropriately to exclude granulocytes, monocytes and doublets which were shown to non-specifically bind both the monoclonal antibodies and the isotype controls. Settings and compensation levels were established using single staining and isotype controls.

3.6.7 Statistical analysis of flow cytometry samples

One way Annova and Tukey pairwise comparison were performed to compare differences between the groups of animals in the percentages of $CD8^+$ T cells expressing a particular surface molecule. As the numbers represent percentages in order to be able to perform

statistical analysis, they had to be first transformed into log ratios using the following logit equation: $\log_{10}$(sample/sample-100)

3.7 Culture of Mϕ and DC

3.7.1 Isolation of $CD14^+$ cells

CD14 positive cells were isolated from blood as described previously (Werling, Hope et al. 1999). Freshly isolated bovine PBMC were incubated with 10 µl per 10^7 cells of anti-human CD14 conjugated superparamagnetic microbeads (Miltenyi Biotec) for 10 min at RT. Unbound microbeads were removed by washing the PBMC twice in ice cold PBSa. To purify CD14 positive monocytes, PBMC were resuspended in 2.5 mls of ice cold sterile FACSflow buffer (3.1 Media formulations) and passed through a MidiMACS LS column (Miltenyi Biotec). The column was then rinsed three times with 2.5mls of FACSflow-BSA buffer and captured cells were eluted by removing the column from the magnet and plunging 5mls of cold TCM. Eluted cells were then washed in TCM and pelleted at 400 x *g* for 5 min at 4^0C. The purified CD14 positive monocytes were enumerated as described 2.2.4 and were resuspended at 10^6cells/ml in either Mϕ or DC media (3.1 Media formulations).

3.7.2 Culture of moncyte-derived Mϕ and DC

Blood derived monocytes to be differentiated into Mϕ-like cells were cultured in teflon pots at for 5 days at 4 x 10^6cells per pot, after 3 days of culture 1ml of fresh Mϕ media was added to each pot. Teflon pots were used instead of 6-well tissue culture plates to prevent the monocyte derived macrophages from adhering therefore increasing cell recovery after 5 days. Monocytes to be differentiated into DCs were cultured in 6-well tissue culture plates (Nalge Nunc International) at 3 x 10^6 cells per well for a total of 5 days, after 3 days 0.5mls of fresh DC media was added to each well.

3.7.3 Harvesting monocyte-derived Mϕ and DC

Monocyte-derived Mϕ were harvested from teflon pots after 5 days of culture by removing the cell suspension and washing the pots three times with ice cold PBS to remove any adherent cells and the washings were added to the cell suspension. The recovered cells were pelleted by centrifugation (400 x *g* for 5 min at 4^0C) and resuspended in infecting media (3.1 Media formulations) for counting.

To recover the monocyte-derived DCs, the non-adherent cells and medium was removed from the plates and 1ml of Cell dissociation fluid (Sigma-Aldrich) was added to each well and the plates were incubated for 20-30 min at 37^0C 5% CO_2. The dislodged cells were removed and each well was washed three times with ice cold PBSa and the cells, together with the washings, were added to previously removed media. Cells were pelleted by centrifugation, resuspended in TCM and counted as described before.

3.7.4 Infection of monocyte-derived Mϕ and DC (BCG, *M. bovis*, *M. avium*)

Harvested monocyte-derived Mϕ and DCs were infected with BCG and *M. avium* at multiplicity of infection (MOI) ratio of ten mycobacteria to one Mϕ or DC and with *M. bovis* at a MOI of 1:1 if not stated otherwise. All infections were performed overnight at 37^0C 5% CO_2. These ratios have previously shown to give similar infection rates (M Thom MSc Thesis). The mycobacteria were thawed, pelleted, resuspended in TCM and sonicated for 3-4min to prevent clumping. Cells were incubated overnight with the mycobacteria in TCM in plastic universals. Figure 3.1 shows that the highest level of proliferation was observed in response to Mϕ infected with BCG at a MOI of 10:1.

3.7.5 Infection of Mϕ with recombinant modified vaccinia Ankara (MVA)

Monocyte-derived Mϕ were cultured for 2 hrs with 0.5 pfu of either MVA-WT or MVA-Ag85 (kindly donated by Sara Gilbert)(McShane, Brookes et al. 2001). The Mϕ were then

washed in TCM and counted. Mϕ were then added to $CD8^+$ T cells at ratio of 1:5 (Mϕ:$CD8^+$ T cell).

3.8 $CD8^+$ T cell sorting experiments

3.8.1 Macs sorting of $CD8^+$ T cells

PBMC were prepared as described before and incubated with anti-CD8 monoclonal antibody labelled with the fluorochrome APC for 10 min at room temperature. PBMC were washed twice with PBS containing 1% BSA and pelleted at 400 x *g* for 5 min. Pelleted cells were resuspended in anti-APC MACS microbeads and incubated for a further 10 min at room temperature. PBMC were than washed twice as before and resuspended in 2.5 ml (10^9 cells) of PBS BSA. The MACs column was attached to the magnet and washed with 2.5 ml of PBS BSA. The labelled cells were applied to the column and the column was washed twice with 2.5 ml of PBS BSA. To elute the cells, the column was removed from the magnet and flushed through with 5 ml of CM into a clean universal tube. The eluted cells were pelleted and counted as described before. Typically the recovered $CD8^+$ T cell population would represent approximately 10-20% of the total PBMC.

3.8.2 Flow cytometry staining of isolated $CD8^+$ T cells

The CD8-expressing cell population in cattle is a heterologous population of cells containing αβ T cells (70-80%), γδ T cells (10-30%) and to a lesser extent NK cells (1-3%). To obtain a population of αβ-T cells, the γδ T cells and NK cells were removed by a process of flow cytometric sorting according to expression of cell surface molecules.

For sorting based upon expression of CD8 and γδ-T cell receptor (TCR1), the CD8 expressing cells were incubated for 10min at RT with a monoclonal antibody (mAb) GB21A (IgG2b), which recognises the bovine γδ T cell receptor. Cells were washed twice with PBS BSA and resuspended with goat anti-mouse IgG2b labelled with the fluorchrome FITC and incubated for 10min at RT.

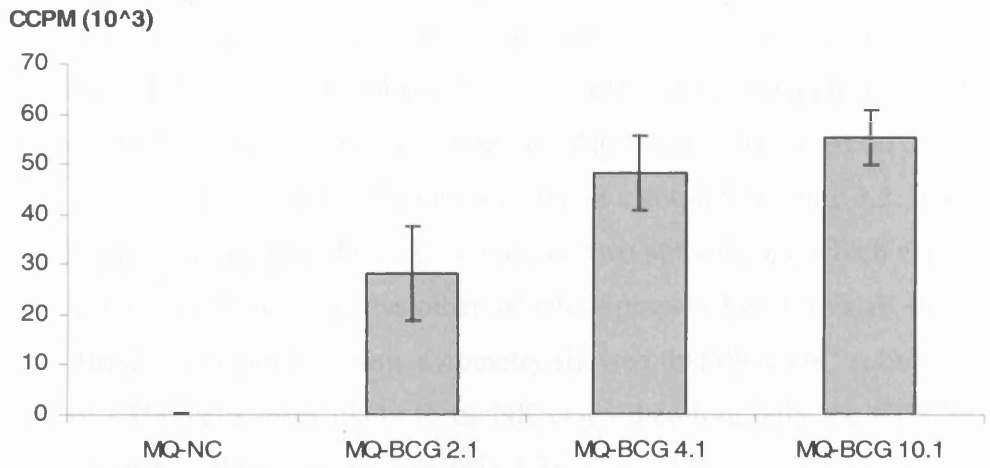
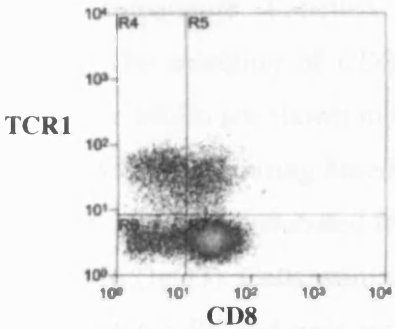


Figure 3.1 Proliferation of $CD8^+TCR1^-CD45RO^+$ T cells after culture with Mϕ infected with BCG at different MOIs. $CD8^+TCR1^-CD45RO^+$ T cells were isolated from blood (7wks) post-vaccination with BCG and stimulated for 5 days with uninfected or infected overnight with BCG at different MOIs 2:1, 4:1 and 10:1 (mycobacteria:APC). APCs were irradiated prior to being added to the $CD8^+$ T cell subsets and uptake of tritiated thymidine was used to measure the extent of proliferation. The mean and standard deviation are shown from triplicate samples. The results shown are from one animal and are representative for the three animals used in the study.



Percentage of each population

R4	$CD8^{lo}TCR1^+$	14.9%
R5	$CD8^{hi}TCR1^+$	10.1%
R6	$CD8^{lo}TCR1^-$	16%
R7	$CD8^{hi}TCR1^-$	60%

Figure 3.2 Two-colour flow cytometric sorting of PBMCs using expression of CD8 and TCR1. PBMC was isolated from blood, stained for expression of CD8 (CC63-APC) and labelled with anti-APC microbeads. CD8 expressing cells were sorted using MACS paramagnetic system and stained with a monoclonal antibody to the γδ T cell receptor. Cells were sorted on the Moflo cell sorter into four subsets; $CD8^{lo}TCR1^+$ (R4), $CD8^{hi}TCR1^+$ (R5), $CD8^{lo}TCR1^-$(R6) and $CD8^{hi}TCR1^-$ (R7). The dotplot shown is representative of the animals used in the cell sorting experiments.

Labelled cells were then washed twice with PBS BSA and resuspended in RPMI supplemented with 10% FCS (3.1 Media formulations) at approximately 1-2 x 10^7 cells/ml for sorting on the Moflo cell sorter. The four populations of cells sorted on the Moflo and the relative percentage of each population is shown in a dotplot in figure 3.2. Expression of CD8 was shown to divide the CD8 population into two subsets, one which expresses high levels of the CD8 molecule and the other which expresses low levels of the molecule. Analysis of these populations by flow cytometry showed that the $CD8^{lo}$ subset contained a population of $CD3^-$ cells most likely to be NK cells, therefore only the $CD8^{hi}$expressing cells were selected for these experiments (Fig 3.3).

For flow cytometric sorting based upon expression of CD8, CD26 and CD45RO, the CD8 expressing cells were incubated for 10 min at room temperature with the murine anti-bovine mAb CC62 (IgG2b) which recognises bovine CD26 and a murine anti-bovine mAb IL-A116 (IgG3) that binds the bovine CD45RO molecule. Cells were washed twice with PBS BSA and resuspended with goat anti-mouse IgG3 labelled with the fluorochrome PE and goat anti-mouse IgG2b labelled with the fluorochrome FITC and incubated for 10 min at room temperature. Labelled cells were then washed and prepared for sorting as described. The selection of $CD8^{hi}$ expressing cells and four populations of cells to be sorted on the Moflo are shown in figure 3.4.

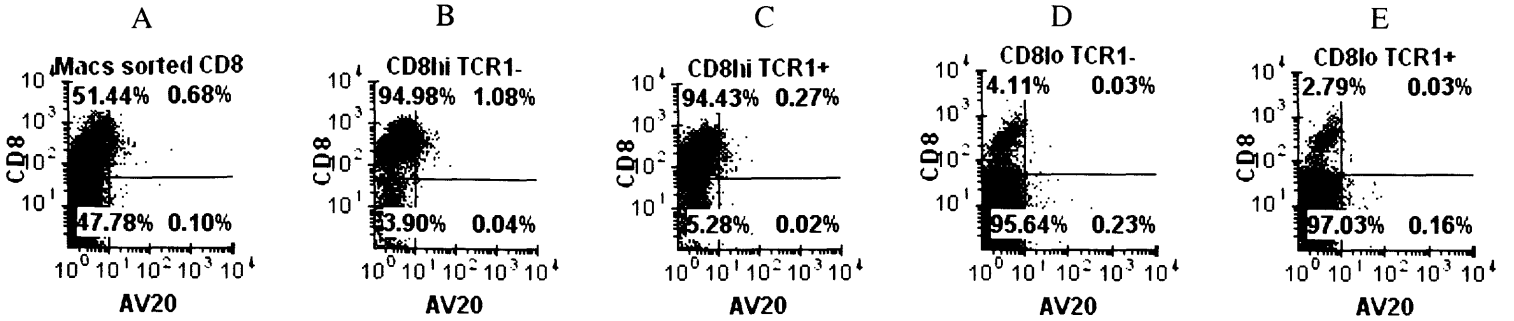
For flow cytometric sorting based upon expression of CD8, TCR1 and CD45RO, the CD8 expressing cells were incubated for 10 min at room temperature with mAbs TCR1 (IgG2b) and IL-A116 (IgG3). Cells were washed twice with PBS BSA and labelled as before with anti-mouse IgG3 PE and goat anti-mouse IgG2b FITC and incubated for 10 min at room temperature. Cells were then washed and prepared for sorting as described. The selection of $CD8^{hi}$cells and dotplots showing the four populations of cells prior and after the sorting process are shown in figure 3.5.

The parameters for the sorting process were set using a panel of controls which included isotype controls, single-colour staining and two-colour staining.

3.8.3 Culture of sorted $CD8^+$ T cell populations

The isolated $CD8^+$ T cell subsets were counted and resuspended in CM at 10^6/ml and 100µl was added per well in a sterile 96-U-well plate with lids (Nunc). Antigen presenting cells were infected as described and were irradiated to prevent any background proliferation. Figure 3.6 demonstrates that irradiation of the APC does not affect the ability of APC to present antigen to the $CD8^+$T cell subsets. The APCs were washed and added to the sorted $CD8^+$ T cells, with PBMC being added at 1:1 and Mϕ or DC at 1:10 or 1:5 (APC:$CD8^+$T cell). It is shown in figure 3.7 that the greatest proliferation was observed when Mϕ and DC were added to T cells at a ratio of 1:5. Concanavalin A (Con A) was used as a positive control and added at 2µg/ml. In most cases unsorted PBMC and MACS sorted CD8 expressing cells were included in these assays as controls. To allow time for the cells to become activated and proliferate they were incubated for 5 days at 37^0C 5% CO_2.

Isotype controls



Staining of sorted subsets for CD3 expression

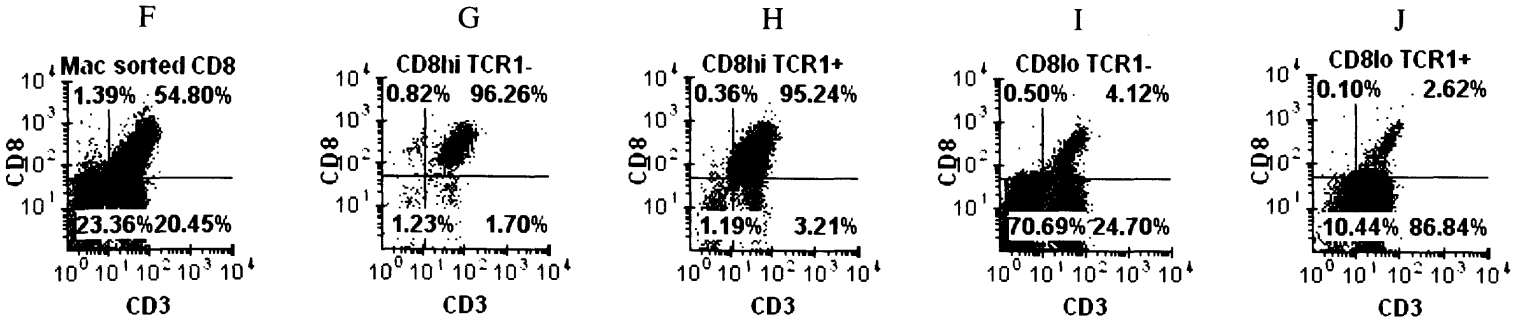
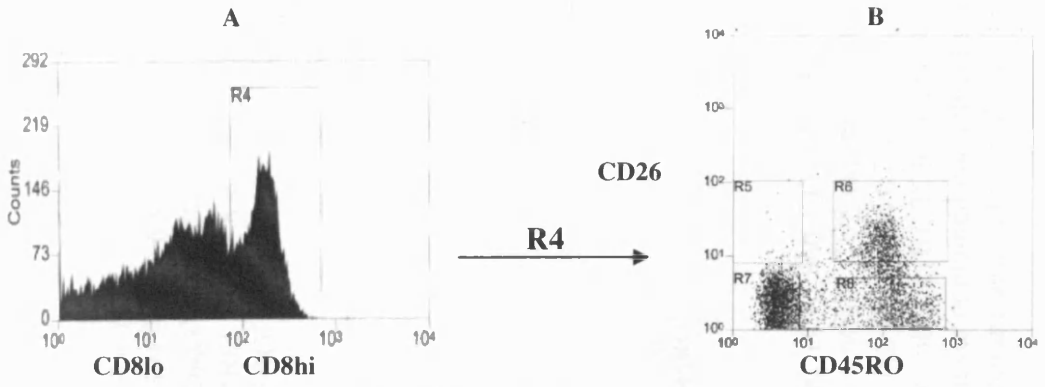


Figure 3.3 Analysis of CD3 expression on sorted CD8 expressing subsets. CD8 expressing cells were isolated from PBMC using MACS^{TM} paramagnetic sorting system and stained with a monoclonal antibody which recognises the γδ T cell receptor (TCR1) prior to being sorted on a Moflo cell sorter. After this second sorting process the following subsets were analysed for expression of the isotype control AV20 (A-E) and CD3 (F-J); $CD8^{hi}TCR1^{-}$ (B+C), $CD8^{hi}TCR1^{+}$ (C+H), $CD8^{lo}TCR1^{-}$ (D+I)and $CD8^{lo}TCR1^{+}$ (E+J). CD8 expressing cells sorted using the MACS system are shown in A and B. The dotplots shown are taken from one animal and are representative of all animals investigated.



R4 $CD8^{hi}$ expression 37%
Sorted into the following:
R5 $CD26^{+}CD45RO^{-}$ 0.6%
R6 $CD26^{+}CD45RO^{+}$ 14.5%
R7 $CD26^{-}CD45RO^{-}$ 48.5%
R8 $CD26^{-}CD45RO^{+}$ 24.4%

Figure 3.4 Three-colour flow cytometric sorting of PBMC based upon expression of CD8, CD45RO and CD26. PBMC was isolated from blood, stained for expression of CD8 (CC63-APC) and labelled with anti-APC microbeads. CD8 expressing cells were sorted using MACS paramagnetic sorting and then stained with monoclonal antibodies to CD26 and CD45RO. Cells were then sorted on a Moflo cell sorter, the cells expressing high levels of CD8 were selected (A) and sorted into four subsets (B); $CD26^{+}CD45RO^{-}$ (R5), $CD26^{+}CD45RO^{+}$ (R6), $CD26^{-}CD45RO^{-}$(R7) and $CD26^{-}CD45RO^{+}$ (R8). The histogram and dotplot shown are representative of the animals used in the cell sorting experiments.

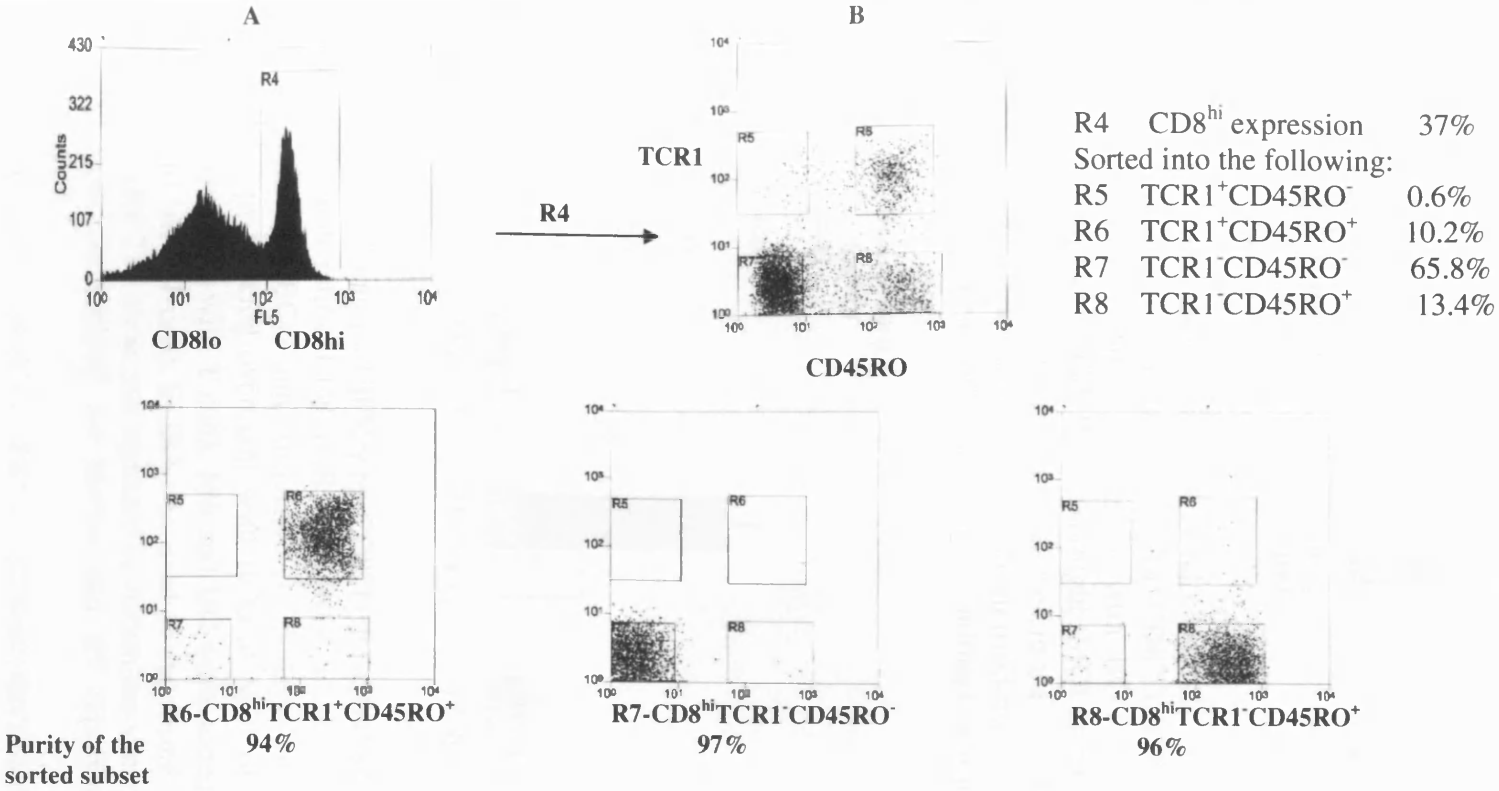


Figure 3.5 Three-colour flow cytometric sorting of PBMC based upon expression of CD8, TCR1 (γδ TCR) and CD45RO. PBMC was isolated from blood, stained for expression of CD8 (CC63-APC) and labelled with anti-APC microbeads. CD8 expressing cells were sorted using MACS paramagnetic sorting and then stained with monoclonal antibodies to TCR1 (γδ TCR) and CD45RO. Cells were then sorted on a Moflo cell sorter, CD8hi expressing cells were selected (A) and sorted into three subsets (B); $TCR1^{+}CD45RO^{-}$ (R5), $TCR1^{+}CD45RO^{+}$ (R6), $TCR1^{-}CD45RO^{-}$(R7) and TCR1-$CD45RO^{+}$ (R8). The $TCR1^{+}CD45RO^{-}$ never contained enough cells to use in the experiments. The purity of the three sorted subsets are shown in B, C and D. The histogram and dotplots shown are representative of the animals studied.

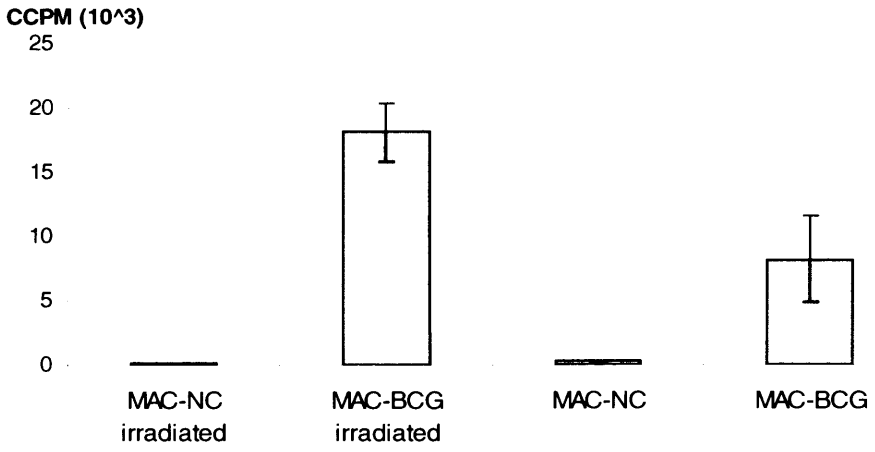


Figure 3.6 Proliferation of $CD8^+TCR1^-CD45RO^+$ T cells after culture with BCG-infected irradiated or non-irradiated Mϕ. $CD8^+TCR1^-CD45RO^+$ T cells were isolated from blood (16wks) post-vaccination with BCG and stimulated for 5 days with uninfected or infected with BCG overnight (MOI of 10:1), irradiated or non-irradiated Mϕ. Uptake of tritiated thymidine was used to measure the extent of proliferation. The mean and standard deviation is shown from triplicate samples. The results are from one animal and are representative for the three animals used in the study.

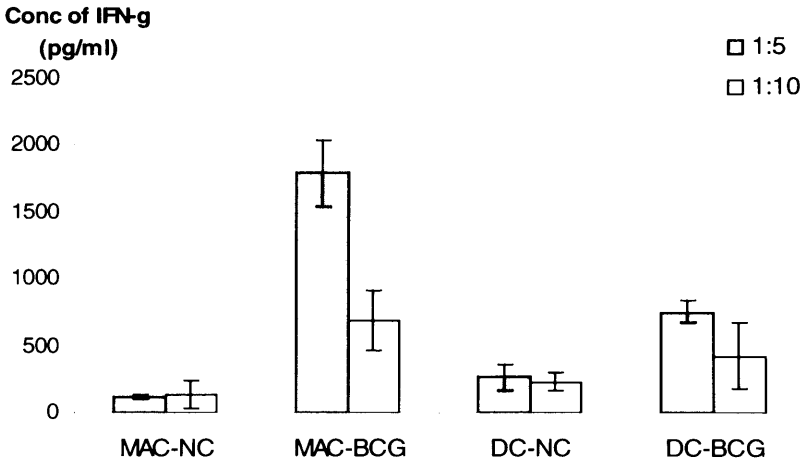


Figure 3.7 Production of IFN-γ by $CD8^+TCR1^-CD45RO^+$ T cells cultured with Mϕ or DC at different ratios. $CD8^+TCR1^-CD45RO^+$ T cells were isolated from blood post-vaccination with BCG and stimulated for 5 days with Mϕ or DC that were either uninfected or infected overnight with BCG at MOI of 10:1 and had been irradiated prior to co-culture with T cells. Mϕ and DC were added to the $CD8^+$ T cells at either 1:5 or 1:10 (APC:T cell). ELISA was used to determine the amount of IFN-γ produced by the T cells. The mean and standard deviation are shown from triplicate samples. The results from one animal are shown and are representative of the three animals investigated.

3.9 Proliferation assay using incorporation of tritiated Thymidine (^{3}H TdR)

All work carried out with radioactive thymidine was performed according to local rules and regulations Act. Incorporation of ^{3}H TdR into the DNA during DNA synthesis is was used as a measure of proliferation. Sorted cells were incubated at 37^{0}C 5%CO_2 and after 5 days, 50-70µl of supernatant was removed for ELISA and 1µCi/well of ^{3}H TdR (Amersham) diluted in RPMI to 50µl was added per well to the 96-well plate and incubated overnight at 37^{0}C 5% CO_2. Plates were harvested onto microfibre filters (Wallac, Leicester) using a Tomtec harvester. Filters were dried and sealed in plastic covers with 5 ml of scintillant (Wallac, Leicester) added. Measurement of ^{3}H TdR incorporation was carried out using a Microbeta Scintillation Counter (Wallac, Leicester)

3.10 ^{51}Chromium (^{51}Cr) release cytotoxicity assay

All work with ^{51}Cr was carried out according to local rules and in designated areas behind lead bricks and all equipment was monitored before and after use with Geiger counter.

Sorted $CD8^+$ T cell subsets ($TCR1^-CD45RO^+$ and $TCR1^-CD45RO^-$) were stimulated for 5 days with BCG-infected monocyte derived Mϕ. The stimulated $CD8^+$ T cells to be used as the effector cells were harvested, counted and resuspended in TCM at 5 x 10^6/ml. In a 96 V-well plate (Nunc), to prepare the top effector to target (E:T) ratio, 200µl of $CD8^+$ T cells was added and from this 1:2 dilutions using CM were made in triplicate to give E:T ratios of 50:1, 25:1, 12:1, 6:1, 3:1, 1.5;1.

The target populations used in this assay were autologous monocyte-derived uninfected and BCG-infected Mϕ. After overnight infection, the Mϕ were washed, counted and resuspended in TCM and 10^6 cells were placed in a 2ml plastic O-ring tube. Cells were pelleted by centrifugation 400 *g* for 5 min, resuspend in 3.7MBq ^{51}Cr (Amersham), placed into lead pots and incubated on a roller for 1.5 hrs at 37^{0}C. The labelled target cells were then washed with TCM three times, counted and resuspended at 10^5/ml. To account for spontaneous release of ^{51}Cr from the target cells, 100µl of labelled Mϕ were added in triplicate to wells containing 100µl of TCM. To measure the maximum

release of ^{51}Cr 100µl of target cells were added in triplicate to wells containing 100µl of 2% SDS (Sigma-Aldrich) in DDQ water. The spontaneous release in all experiments was less than 10% of the maximum release of ^{51}Cr. The target cells (100µl) were added to the previously prepared effector cells. The 96-well plate was centrifuged with the brake off at 300 *g* for 3 min and incubated at 37^0C 5% CO_2 for 4.5 hrs. After which 25µl of the supernatant was removed from each well and pipetted onto a filter mat and the filter mat was allowed to dry overnight. For counting the filter mat was placed inside a plastic mat (Perkin Elmer, Wallac) and 10mls of scintillant (Perkin Elmer, Wallac) was added and dispersed evenly over the filter map using a roller, before being placed into a cassette and counted on the Beta-plate counter (Wallac).
The amount of ^{51}Cr released from the target cells was equated to the percentage of target cells lysed by the effector cells using the following calculation

$$\% \text{ Specific } ^{51}Cr \text{ release} = \frac{\text{CPM test} - \text{CPM spontaneous}}{\text{CPM maximum} - \text{CPM spontaneous}} \times 100$$

3.11 Measurement of IFN-γ in the cell assay supernatants using ELISA

Previously published methods for the detection of bovine cytokines by ELISA were used (Hope, Kwong et al. 2000). Briefly, 96-well flat-bottomed clear microplates (Nunc) were coated overnight with 100µl/well of a monoclonal antibody to bovine IFN-γ (CC302) at 1µg/ml diluted in coating buffer (3.1 Media formulations). To remove unbound mAb, the plate was washed five times with washing buffer (3.1 Media formulations) using an automated plate washer (Skatron Skanwasher 300; Skatron Instruments). Non-specific binding sites were blocked by incubating the plates with blocking buffer (3.1 Media formulations) for 1-2hrs at room temperaure. The plates were then washed as before and samples were diluted as appropriate, in blocking buffer and plated out in triplicate. A standard curve was prepared by making three-fold dilutions in blocking buffer of recombinant bovine IFN-γ obtained from supernatant of COS-7 cells expressing plasmid encoding bovine IFN-γ cDNA. The plates were incubated with samples and standards for 1 hr and washed as before. To detect bound cytokine, plates were incubated for 1 hr with 100 µl/well biotinylated monoclonal antibody to bovine IFNγ (CC330) at 2 µg/ml diluted in blocking buffer. The plates

were washed as described previously and incubated for 45 min with Streptavidin conjugated to reporter enzyme horseradish peroxidase (SA-HRP; Amersham plc) diluted 1:500 in reagent diluent (3.1 Media formulations). Plates were then washed and developed with 100 µl/well of 3,3`5,5`-tetramethylbenzidine (TMB) substrate solution (3.1 Media formulations). The photochemical reaction was stopped with 50µl/well of 4M H_2SO_4. Absorbance was measured at 450nm (maximal absorbance of TMB) and at 690 nm to exclude non-specific background using Anthos Lucy 1.0 microplate reader (Anthos Labtech Instruments GmbH). Specific absorbance was determined by subtracting absorbance at 690 nm from that at 450 nm. The amount of cytokine present in samples was calculated from the standard curve constructed using the titration of the cytokine standard of known concentration.

3.12 Analysis of survival of mycobacteria inside Mϕ

$CD8^+$ T cell subsets ($TCR1^-CD45RO^+$ and $TCR1^-CD45RO^-$) were cultured with BCG-infected, *M. bovis*-infected or uninfected Mϕ for 5 days. Cells were lysed by addition of 0.1% Triton X-100 in SQW. Cells were then incubated at 37^0C for 15 min and serial dilutions were made in triplicate in 7H9 media from the cell lysate supernatant and plated onto 7H10 (BCG) and 7H11 (*M. bovis*) plates. The plates were incubated at 37^0C for 3 weeks and the number of viable mycobacteria present after co-culture of infected Mϕ and $CD8^+$ T cells was determined.

3.13 Molecular Biology Techniques

3.13.1 Isolation of RNA

$CD8^+$ T cell subsets were harvested from 96-wells plates after being cultured for 5 days with uninfected or BCG-infected monocyte-derived Mϕ. Cells were pelleted by centrifugation (400 *g* for 5 min) and lysed by addition of GTC (Appendix I). Cell suspensions were then homogenised using QIAshredder columns (Qiagen) according to the manufacturer's instructions. RNA was isolated using the RNeasy Mini kit (Qiagen) again according to the manufacturers' instructions. Briefly, cell lysates were diluted in an equal volume with 70% molecular grade, DNA-free, RNA-free ethanol (VVR International Ltd) in Super Q water (SQW) (IAH-media services). Cell lysates were added to a Minispin column (Qiagen) and centrifuged at 8000 *g* for 15 s. The flow-through was discarded and the column was washed with 700µl of RW1 buffer and centrifuged at 8000 x *g* for 15 s. Both the flow through and collection were discarded and the column was transferred to a new RNeasy column. The column was then washed twice with 500µl of the RPE buffer and the column was centrifuged as above. After the second wash the column was centrifuged for a further 2 min at 8000 x g to dry the silica-gel membrane of the column. To prevent any carry over of ethanol the RNeasy column was transferred to a new collection tube and centrifuged at full speed (13 000 x *g*) for 1 min. To elute the RNA the column was then transferred to a new collection tube and 30µl of RNase-free water was applied to the column and the column was centrifuged at 8000 x g for 1 min. To increase the yield of the RNA a further 30µl of RNase-free water was added to the column.

The recovered RNA was treated with RNase-free DNase to remove any contaminating DNA using DNA-free™ kit (Ambion) according to manufacturer's instructions. Briefly, 6µl of DNase I buffer and 1µl of rDNase I was added to the RNA, mixed gently and incubated at 37^0C for 30 min. To stop the reaction 6µl of DNase Inactivation Reagent was added and incubated at RT for 2 min during which the sample was mixed occasionally. The sample was then centrifuged at 10 000 x *g* for 1.5 min to pellet the DNase inactivation reagent and the RNA was carefully pipetted into a sterile eppendorf tube.

3.13.2 cDNA synthesis

Complementary DNA (cDNA) was synthesised from RNA using the Reverse Transcription System kit (Promega Ltd) which utilises the reverse transcriptase of the avian myeblastoma virus (AMV). The reaction was carried out in an autoclaved 0.2 ml thin-walled PCR tube (Abgene) at a final volume of 40 µl.

For each reaction:

COMPONENT	AMOUNT (µl)
25mM Magnesium chloride ($MgCl_2$)	8
Reverse Transcription Buffer (10x)	4
10mM dNTP mix	4
Recombinant RNasin Ribonuclease Inhibitor	1
AMV Reverse Transcriptase (High conc)	1.5
Oligo $(dT)_{15}$ Primer	2
mRNA	(200ng)
Sterile RNase free water	make up to final volume 40µl

The sample was mixed and centrifuged at 8000 x g for 5 s and cDNA synthesis was performed using iCycler PCR (Biorad) at 70^0C for 10 min, 42^0C for 1 hr, 95^0C for 5 min and 4^0C for 5 min. The cDNA was stored at -20^0C until it was required for RT-PCR.

3.13.3 Quantification of DNA/RNA by spectrophotometry

The concentration of cDNA and RNA was determined by measuring the optical density at wavelengths 260nm and 280nm using spectrophotometer (Ultraspec 2100 Pro, Biochrom) with the background set at 320nm. The DNA/RNA was measured at two different dilutions in RNase free-water (Sigma) to achieve a more accurate concentration and RNase free-water was used as the initial reference reading. The purity of the DNA/RNA was determined by reading the OD at 260/280nm. If the sample showed a reading of greater than 1.8 this indicated a low protein contamination

and sufficient purity of the sample. The DNA/RNA concentration was calculated by the Spectrophotometer and given in µg/µl.

3.13.4 Polymerase Chain Reaction

PCR amplification of cDNA was performed using Taq DNA polymerase (Invitrogen) derived from *Thermus aquaticus*. This enzyme is heat-stable and has 5'-3' DNA polymerase activity and a 5'-3' exonuclease activity but lacks a 3'-5' exonuclease activity.

For each reaction:

COMPONENT	AMOUNT (µl)
50mM Magnesium chloride ($MgCl_2$)	1.5
10X PCR buffer minus Mg^{++}	5
10mM dNTP mix (Promega)	1
Taq DNA polymerase	0.5
Upstream primer	2
Downstream primer	2
Template cDNA	(1µg)
Sterile RNase free water	make up to final vol 50µl

For each set of reactions for a pair of primers a negative control in which the cDNA was replaced with RNase free water was included. The house-keeping gene β-actin was included in the RT-PCR reactions to ensure that all samples contained comparable amounts of cDNA and produced bands of similar intensity at 270bp.

PCR reactions were carried out in thin walled microtitre plates with dome-shaped caps (Abgene) using an iCycler PCR machine (Bio-Rad). The PCR conditions used were as follows:

1 Cycle:	Denaturing 92^0C for 2 min
30 Cycles:	Denaturing 92^0C for 1 min
	Annealing 58^0C for 45 sec
	Extension 72^0C for 45 sec
1 Cycle:	Extension 72^0C for 10 min
	Hold at 4^0C

The extension times were calculated based on a Taq DNA polymerase extension rate of 1kb per minute.

Table 3.13.1 Primers for RT-PCR

All primers were obtained from MWG Biotech.

Product	Primer Sequence (5'- 3')	Reference
β-actin	CCAGACAGCACTGTGTTGGC GAGAAGCTGTGCTACGTCGC	Collins et al 1996
CCR7	CAAGATGAGGTCACGGACAA TTGCTGATGAGAAGGACACG	Dirk Werling (RVC)
Perforin	GATGCCAACTTCGCCGCCCA TGTCAGTCACGTACTTGCTC	(Endsley, Furrer et al. 2004)
Bolysin	CTGCTGCTCCAAGGAGAAGA GCAGTGGAGGGAGTTTGGT	(Endsley, Furrer et al. 2004)

3.13.5 Agarose Gel Electrophoresis of PCR products

The size and presence of a PCR product was determined by agarose gel electrophoresis. Gels were prepared with 1 % agarose (Helena Biosciences) in 1 x TBE buffer (Gibco). Ethidium bromide (1 μg/ml) was added to the gel and running buffer to visualise the DNA bands. The samples were mixed with loading buffer (3.1 Media formulations) at a

ratio of 5:1 and loaded onto the same gel together with a 1Kb DNA ladder (Ambion). Electrophoresis was carried out at 100v for the appropriate length of time. The DNA bands were visualised using a UV Trans Illuminator.

3.14 Immuno-fluorescence

3.14.1 Preparation of sections

Prescapular lymph nodes were removed at necropsy from animals that had been previously vaccinated with BCG and skin-tested 3 days prior to necropsy with either PBS or 10^6 BCG. Approximately 1 cm^3 sections were removed from the lymph node and embedded in optimal cutting temperature (OCT) compound (Tissue Tek) in an aluminium foil cube and rapidly placed in liquid nitrogen to freeze and stored at -70^{0}C. For preparation of sections, frozen tissue blocks were allowed to warm to -20^{0}C and serial sections were cut using a cryostat onto snowcoat slides (Surgipath). Freshly cut sections were fixed in ice-cold acetone for 5 min and stored short-term at -20^{0}C.

3.14.2 Immuno-fluorescence staining of sections

Frozen slides were allowed to warm to RT before being fixed in ice-cold acetone for 5 min. Slides were allowed to dry and a Dakocytomation hydrophobic pen (Dakocytomation, CA) was used to carefully draw a circle around the section to prevent the solutions from running. To block any non-specific binding of mAb, sections were incubated with 300µl of 5% goat serum (Sigma-Aldrich) diluted in PBSa for 20-30 min at RT. The goat serum was removed and slide was incubated with 200µl of primary mAb (CC63 10µg/ml, MM1A 10µg/ml and perforin 2 µg/ml) for 20-30 min. The excess primary mAb was removed from the slide by washing in a PBSa bath. The slide was then incubated with 200µl of secondary fluorescently labelled mAb diluted at appropriate concentration for 20-30 min, after which the slide was washed in PBS as before. Slides were removed from the PBS bath one at a time then dipped into super Q water and excess water was removed from the section before adding one drop of mounting media (Dakocytomation, CA). A cover slip (Chance Propper Ltd, West Midlands) was then carefully placed onto the section to prevent any bubbles from

forming between the section and cover slip. The slides were then stored at 4^0C overnight before being analysed under a confocal microscope.

3.14.3 Analysis using Confocal microscope

Settings and compensation levels were set using single stained controls and isotype controls. The argon ion laser was used for analysis of antibodies labelled with FITC (488nm), the krypton laser for TritC (568nm) and the HeNe laser was used for analysis of antibodies labelled with APC (633nm). The lasers were set at 488nm (38%), 568nm (71%) and 633 (33%) with the compensation levels shown in brackets.

CHAPTER FOUR: RESULTS

Expression of cell surface and effector molecules by bovine $CD8^+$ T cells

4.1 Introduction

Ageing is associated with an increased susceptibility to infectious, neoplastic and autoimmune disease thought to be due to an impaired ability to mount an effective humoral and cellular immune response. This impairment in aged individuals has been attributed to an evident change in the composition of the T cell compartment. In particular, a decrease in the proportion of T cells expressing a naïve phenotype and an accumulation of T cells expressing a memory phenotype has been observed in the elderly individuals (De Paoli, Battistin et al. 1988; Utsuyama, Hirokawa et al. 1992). This change in the balance of T cell subsets is thought to be a consequence of the involution of the thymus which starts to degenerate in humans from the age of 1 yr, resulting in individuals older than 50 having less than 20% of tissue able to carry out thymopioesis (Steinmann 1986; Mackall, Fleisher et al. 1995; Aspinall and Andrew 2000). The reduced output of naïve T cells from the thymus in elderly individuals means that the number of antigens that can be recognised will decrease and the probability of a naïve T cell meeting its antigen will be reduced. It is also widely recognised that with age comes an increase in the exposure to antigens resulting in an accumulation of memory cells within an individual. It is thought that the total number of T cells does not change significantly over an individual's lifetime (young adults possess 2-3 x 10^{11}) and that the T cell pool is a finite space (Hulstaert, Hannet et al. 1994; Douek, McFarland et al. 1998; Haase 1999). In order to accommodate the increase in the memory T cell pool it is thought that old memory T cells maybe replaced by recently formed memory T cells and also that the new memory T cells may fill the space left by the decrease in the number of naive T cells.

Expression of a number of cell surface molecules have been used in the human and murine studies to distinguish between naive, effector and memory $CD8^+$ T cells. Originally expression of CD45RA was attributed to naïve unprimed T cells and CD45RO to memory T cells (Akbar, Terry et al. 1988; Beverley, Merkenschlager et al. 1988; Merkenschlager

and Beverley 1989). This simplistic definition using expression of CD45RA and CD45RO to identify naïve and memory cells was complicated by studies in humans showing that a proportion of memory $CD8^+$ T cells down-regulate CD45RO expression and re-express CD45RA. These $CD8^+CD45RA^+$ T cells are resistant to apoptosis, exhibit effector functions similar to primed cells and are present at a high frequency in the elderly and individuals with persistent infections (Okumura, Fujii et al. 1993; Nociari, Telford et al. 1999; Baars, Ribeiro Do Couto et al. 2000; Faint, Annels et al. 2001; Kuijpers, Vossen et al. 2003). It is now recognised that the memory T cell population is heterogeneous in terms of expression of cell surface molecules, effector function and homing capacity. Expression of CCR7 and CD45RA has been used to divide the different subsets of $CD8^+$ T cells into naïve T cells ($CD45RA^+CCR7^+$), effector or RA-primed ($CD45RA^+CCR7^-$), effector memory ($CD45RA^-CCR7^-$) and central memory ($CD45RA^-CCR7^+$)(Hamann, Baars et al. 1997; Sallusto, Langenkamp et al. 2000; Sallusto, Geginat et al. 2004). The two populations of memory T cells described as central and effector memory T cells were defined by their migratory capacity and effector capabilities. Central memory cells are $CD45RO^+CD62L^+CCR7^+$, home to the lymph nodes (LN) (Bromley, Thomas et al. 2005) and display limited immediate effector function and upon exposure to antigen they proliferate and differentiate into effectors cells. Effector memory T cells are described as being $CD45RO^+CD62L^-CCR7^-$, migrate into non-lymphoid tissue and express immediate effector functions upon antigen stimulation (Sallusto, Geginat et al. 2004).

Studies in humans investigating changes with age in the proportions of $CD8^+$ T cells subsets showed a decrease with age in the proportion of cells expressing CD45RA and an increase in the proportion of $CD8^+$ T cells expressing CD45RO (Aldhous, Raab et al. 1994; Wallace, Zhang et al. 2004). A report by Hong et al 2004 (Hong, Dan et al. 2004) demonstrated that the proportions of $CD8^+$ T cell subsets change with age, expression of CD45RA and CCR7 was used to distinguish between naive ($CD45RA^+CCR7^+$), effector ($CD45RA^+CCR7^-$), effector memory ($CD45RA^-CCR7^-$) and central memory ($CD45RA^-CCR7^+$) $CD8^+$ T cells (Hamann, Baars et al. 1997). It was shown in blood that there was decrease in the percentage of naïve $CD8^+$ T cells and an increase in the percentage of effector and effector memory $CD8^+$ T cells in elderly individuals (≥65yrs) compared to young individuals (≤40yrs). Interestingly, no significant difference was observed in the

frequency of the central memory $CD8^+$ T cell subsets between the two age groups (Hong, Dan et al. 2004). It is suggested that the observed changes with age in the frequency of the $CD8^+$ T cell subsets may contribute to the age-associated alterations in T cell function.

The expression of molecules involved in co-stimulation CD28 and CD27 on $CD8^+$ T cells is reported to decline with age in healthy individuals and is reduced in individuals with chronic infections or diseases (Fagnoni, Vescovini et al. 1996; Effros 1997; Brzezinska, Magalska et al. 2004). CD28 and CD27 are primarily expressed by naïve T cells and are required for optimal antigen stimulation. Expression of these molecules is thought to be lost upon chronic antigen stimulation as $CD28^-$ and $CD27^-$ $CD8^+$ T cells have shorter telomeres than their counterparts suggesting that these cells have undergone a number of cell divisions. An influenza infection model in mice demonstrated that flu-specific $CD8^+$ T cells only lost expression of CD27 after secondary infection (Baars, Sierro et al. 2005). In addition, $CD8^+CD28^-$ and $CD8^+CD27^-$ T cells were found to be cytotoxic, containing high levels of cytotoxic mediators such as perforin and granzymes (Azuma, Phillips et al. 1993; Fagnoni, Vescovini et al. 1996; Baars, Sierro et al. 2005)

Bandres et al demonstrated a direct correlation in humans between increases in the percentage of $CD8^{hi}CD28^-CD57^+$ T cells and the percentage of $CD8^+$ T cell expressing IFN-γ with age (Bandres, Merino et al. 2000). The $CD8^+CD27^-$ and $CD8^+CD28^-$ T cells are subdivided by expression of CD45RO and some of these cells were found to have re-expressed CD45RA (Kuijpers, Vossen et al. 2003). These findings indicate that in humans the proportion of effector and/or effector memory cells increases with age.

Although a multitude of studies have been published on the definition of naïve, effector and memory $CD8^+$ T cells in human and mice, to date, little is known about the different $CD8^+$ T cell subsets present in cattle. The available literature shows that $CD8^+$ cells in bovine PBMC can be divided into four populations based upon expression of CD45R and CD62L (Howard, Sopp et al. 1992). In addition, it has been reported that in animals immune to the protozoan parasite *Theileria parva* the responding $CD8^+$ cells are found within both the $CD45RO^+$ and $CD45RO^-$ subsets (Bembridge, MacHugh et al. 1995). Thus, the main focus of this chapter is to define naïve, effector and memory $CD8^+$ T cells in cattle using expression of cells surface molecules and effector molecules previously

studied in human and murine studies. It has been well documented in humans that the proportions of $CD8^+$ T cell subsets change with age. Therefore, the percentage of $CD8^+$ T cells expressing cell surface and effector molecules in blood from animals of different ages was investigated. Studies in human and mice have shown that different subsets of memory $CD8^+$ T cells exhibit different patterns of re-circulation with effector memory cells residing in non-lymphoid tissues and central memory cells in LN (Sallusto, Lenig et al. 1999; Masopust, Vezys et al. 2001). Both memory subsets together with naïve and effector T cells are known to be present in blood. Therefore expression of cell surface and effector molecules by $CD8^+$ T cells in blood is compared with that in LN and lung (BAL).

4.2 Expression of surface molecules on bovine $CD8^+$ T cells present in blood, LN and BAL

To investigate the different cell-types that express the CD8 co-receptor in cattle, PBMC and cells isolated from LN and BAL were stained with mAbs to CD8, CD3 and the γδ T cell receptor (TCR1). The first gate (R1) was placed around a defined population of cells thought to be resting lymphocytes. Within the lymphocyte population the $CD8^+$ cells were gated and analysed for expression of TCR1 and CD3. The population of cells present in cattle which express the CD8 co-receptor was found to be heterogeneous; comprising of γδ T cells (5-30%), a population of cells which lacks the CD3 molecule most likely to be NK cells (0.1-3%) and a population of cells that expresses CD3 but lacks expression of the γδ TCR which are thought to be the αβ $CD8^+$T cells (Fig 4.2.1). In figures 4.2.1 and 4.2.2 it is demonstrated that the frequency of $CD8^+$γδ T cells ($TCR1^+$) increases in the blood with age. A small increase is apparent in the percentage of $CD8^+TCR1^+$T cells present in the BAL when compared to the LN (Fig 4.2.1 and Fig 4.2.2). No significant differences were observed in the percentages of $CD3^+CD8^+$ T cells with age or location.

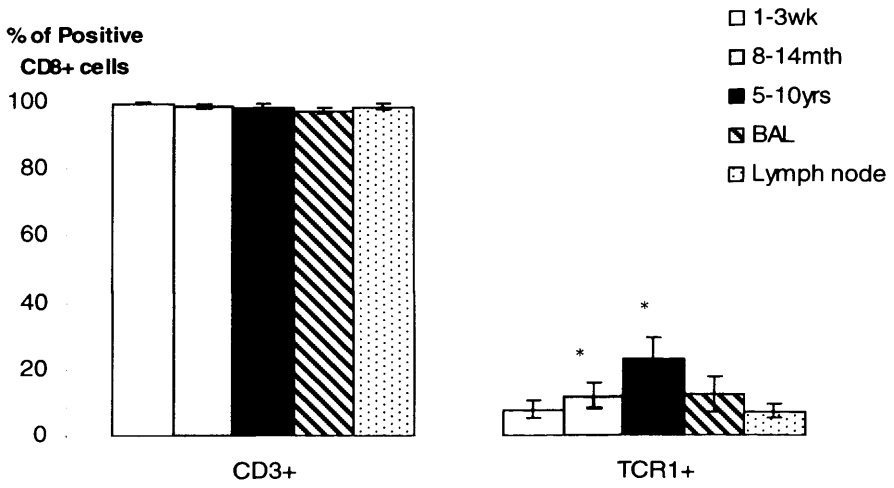


Figure 4.2.1 The heterogeneous nature of the $CD8^+$ cell population in cattle. Flow cytometry was used to analyse the expression of CD3 and the γδ T cell receptor (TCR1) on CD8 expressing cells present in PBMC from animals in the three age groups (1-3wks, 8-14mth, 5-10yrs) and on cells present in LN (prescapular, submandibular, parotid, retropharyngeal) and in BAL from lungs. The mean and standard deviation is shown of at least three animals per group*$p<0.01$.

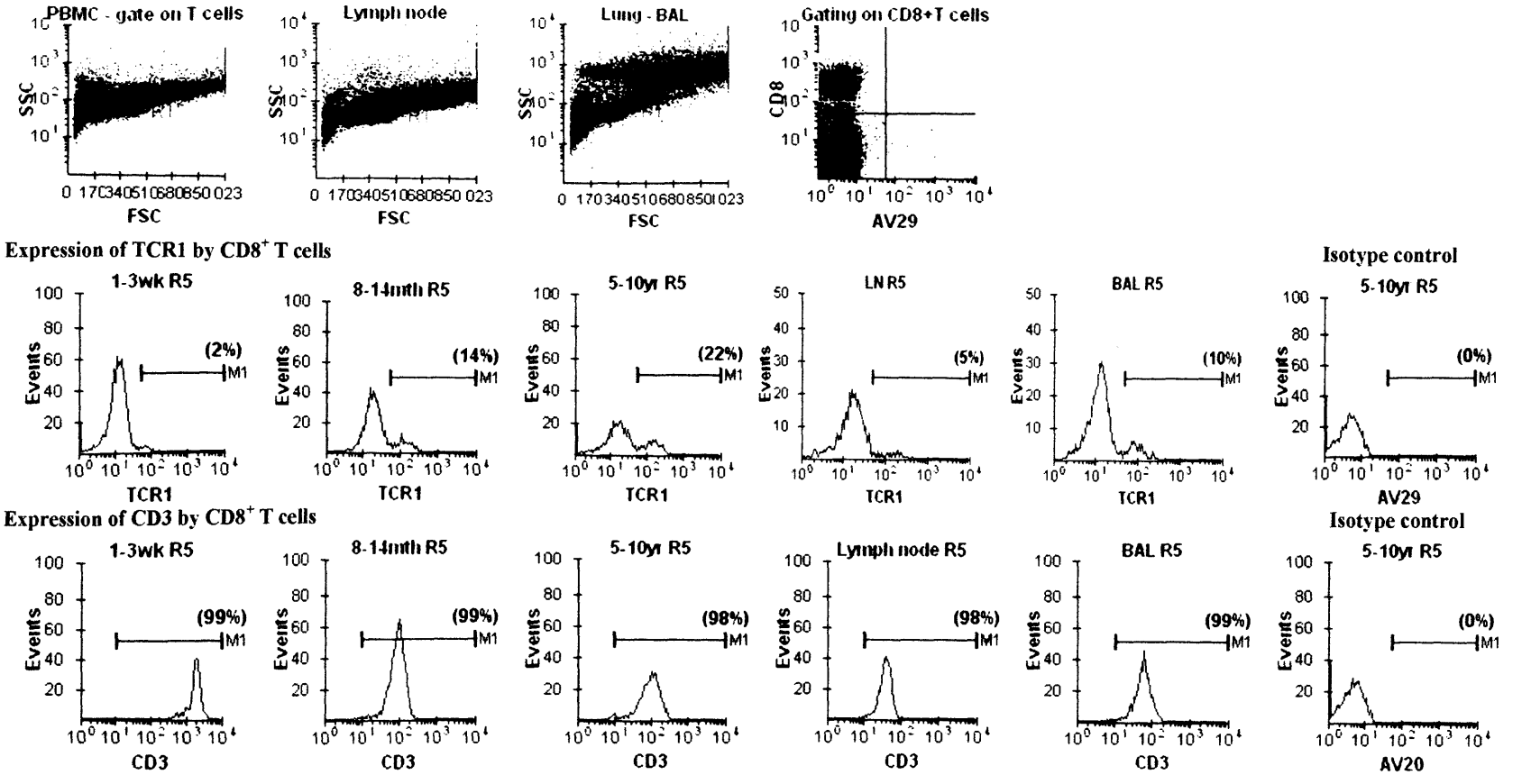


Figure 4.2.2 Differences in the expression of CD3 and TCR1 on $CD8^+$ T cells with age and anatomical location. PBMC and cells from the LN and BAL were stained with antibodies to CD8, CD3 and γδ T cell receptor (TCR1). Gates were placed to select the T cell region (R1) based on FCS and SSC scatter plot and also around the CD8 expressing cell population (R5) and cells within these two regions were analysed for expression of CD3 and TCR1. Representative histograms are shown from one animal from each group.

The aim of this study is to investigate the development of $CD8^+\alpha\beta$ T cell responses in bovine TB. It is shown in figures 4.2.1 and 4.2.2 that in cattle the $CD8^+\gamma\delta$ T cells can account for up to 30% of the total $CD8^+$ cell population. The maximum percentage of NK cells in the $CD8^+$ population was found to be 2% (Fig 4.2.1 and 4.2.2). In the materials and methods, figure 3.3 demonstrates that by gating on the cells which express high levels of the CD8 co-receptor it is possible to exclude the majority of the $CD3^-$ cells. Therefore, in order to analyse the pattern of expression of surface molecules present on $\alpha\beta$ $CD8^+$ T cells, flow cytometry was used to exclude the cells expressing the $\gamma\delta$ TCR and gate on the $CD8^{hi}TCR1^-$ T cells for analysis of expression of surface markers and effector molecules.

To identify surface molecules differentially expressed by subsets of $CD8^{hi}TCR1^-$ T cells, PBMC were obtained from animals from the three age groups (1-3wks, 8-14mth, 5-10yrs). Cells were also isolated from LN and BAL and were stained with antibodies to CD8, TCR1 and one of the following surface molecules CD45RO, CD45RA, CD62L, CD27, CD28, CD25 and CD3.

It was found that the percentage of $CD8^+TCR1^-$ T cells present in PBMC that express CD62L, CD45RA, CD27 and CD28 decreased with age (Fig 4.2.3A and 4.2.4). Whilst the percentage of $CD8^{hi}TCR1^-$ T cells expressing CD45RO, CD25 and CD26 increased with age (Fig 4.2.3A and 4.2.4). The observed changes suggest an age-related increase in the proportion of $CD8^{hi}TCR1^-$ T cells that express an effector/memory phenotype ($CD45RO^+$, $CD62L^-$, $CD27^-$, $CD28^-$) and a decrease in the proportion of cells expressing surface molecules associated with a naïve phenotype ($CD45RA^+$, $CD62L^+$, $CD27^+$, $CD28^+$).

The $CD8^{hi}TCR1^-$ T cells present in LN predominantly expressed CD62L, CD45RA and lacked CD45RO and CD25. Interestingly, a small population of $CD8^{hi}TCR1^-$T cells present in the LN expressed CD45RO suggesting the presence of a memory T cell population in bovine LN. The results in figures 4.2.3B and 4.2.5 demonstrate that almost all of the $CD8^{hi}TCR1^-$ T cells present in BAL express CD45RO and predominantly lack expression of CD62L and CD45RA.

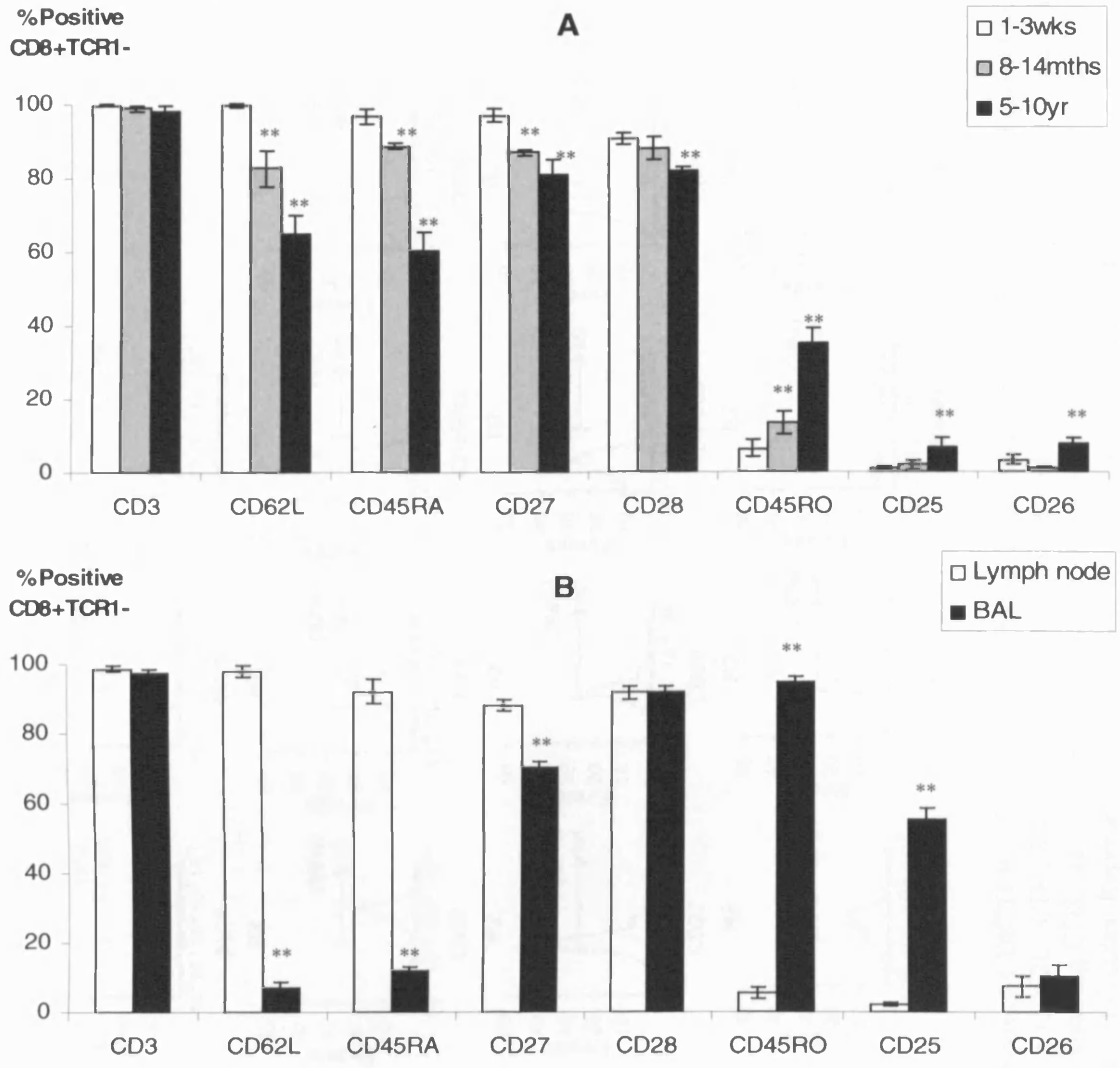


Figure 4.2.3 Differences in the surface phenotype of $CD8^{hi}TCR1^{-}$ T cells in blood with age and between LN and BAL. Three-colour flow cytometry was used to investigate the expression of surface molecules on $CD8^{hi}TCR1^{-}$ T cells present in PBMC from animals from three different age groups (1-3wk, 8-14mth and 5-10yrs)(A) and cells present in the BAL and LN (B). The mean and standard deviation are shown for each group. Each group is comprised of at least three animals. Differences between adjacent age groups or between the LN and BAL that are statistically significant is shown on the older of the two groups or on the BAL **$p<0.001$.

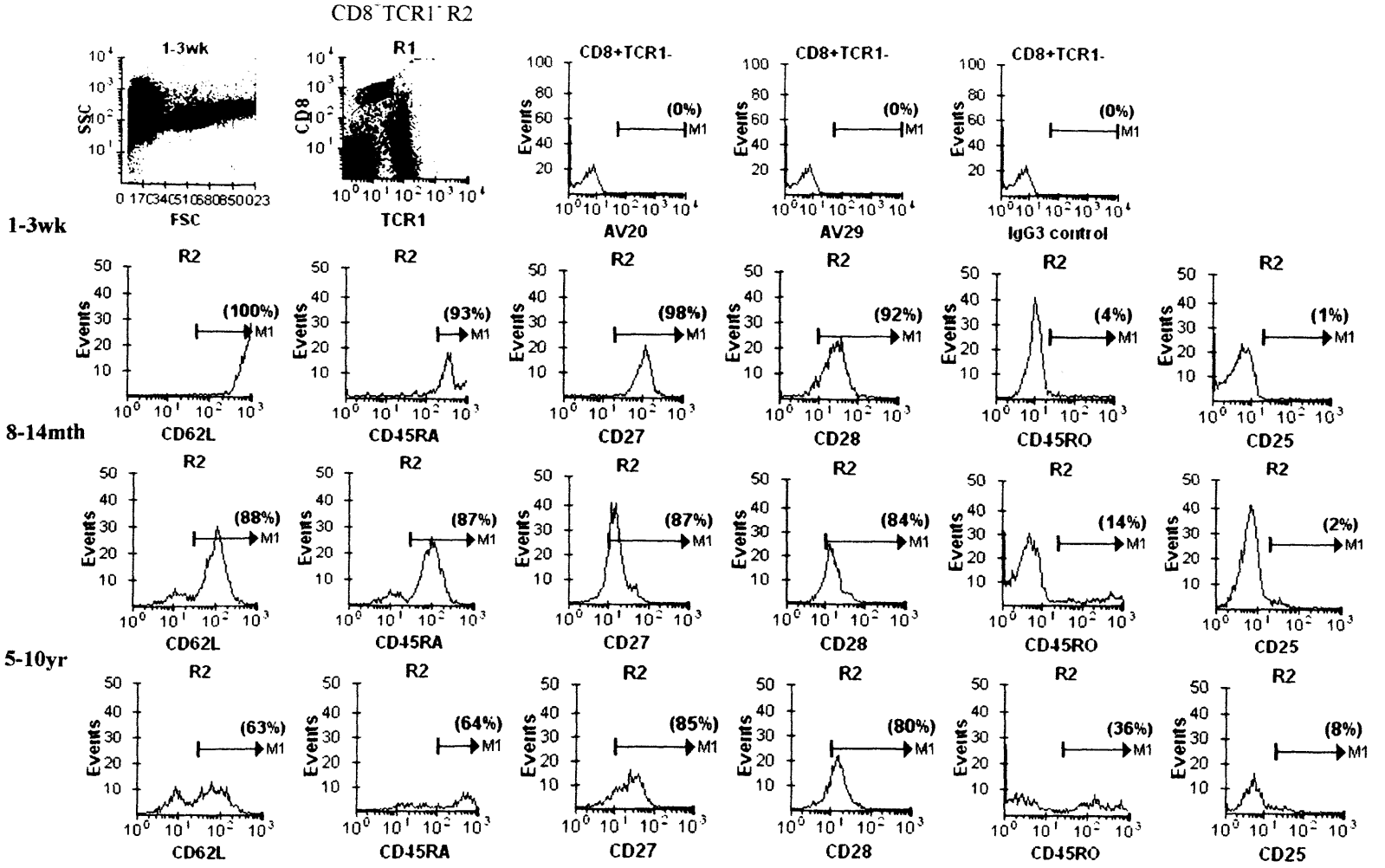


Figure 4.2.4 Age-related differences in the proportions of $CD8^{hi}TCR1^{-}$ T cell subsets in blood. PBMC were isolated from blood and stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD27, CD28 and CD25. A gate was drawn around the lymphocyte fraction (R1) and was applied to dotplot showing CD8 against TCR1 and a further gate was drawn around the $CD8^{hi}TCR1^{-}$ cells (R2) and these cells were analysed for expression of surface molecules. Representative histograms are shown for one animal per group.

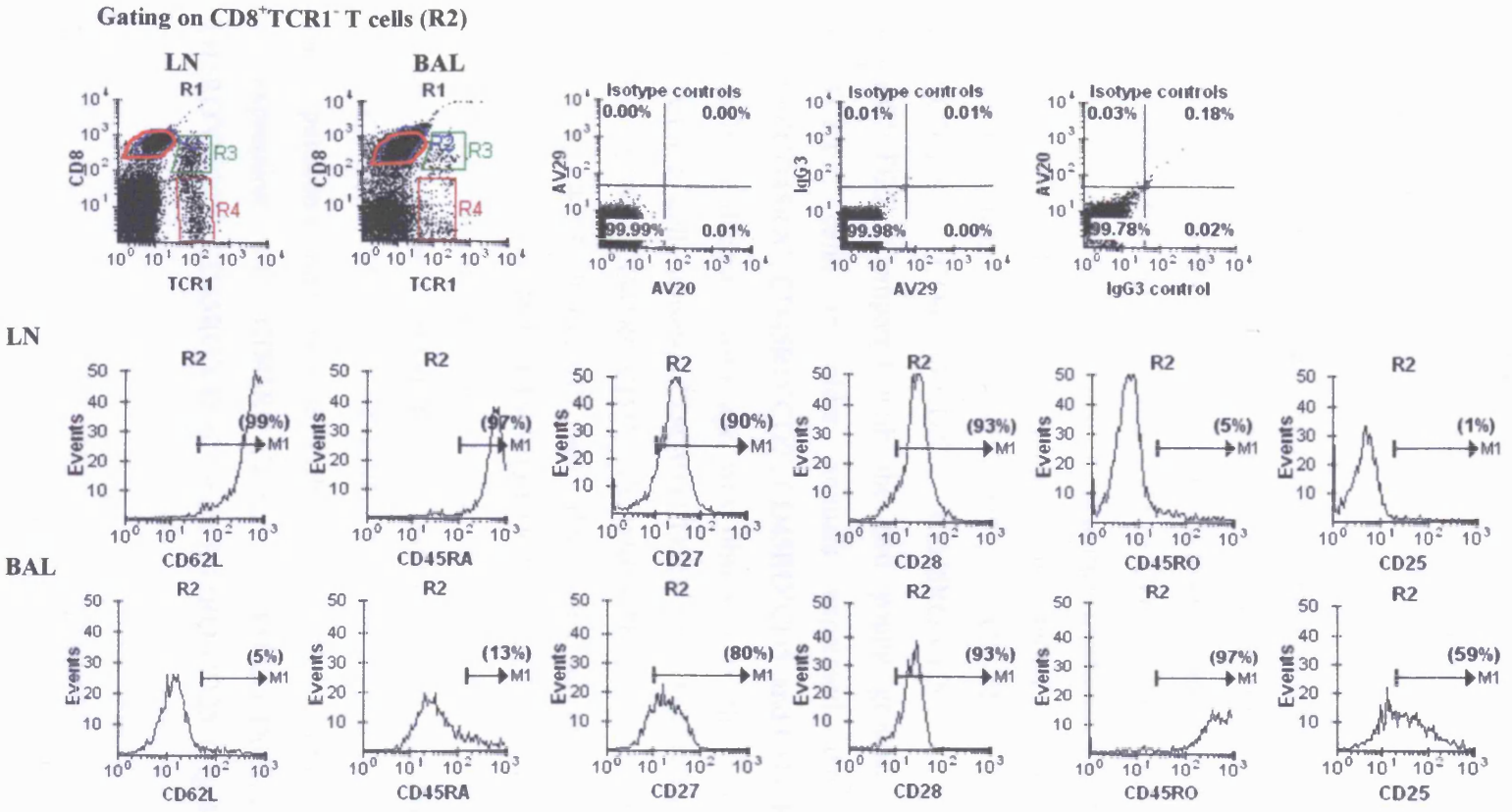


Figure 4.2.5 Differences in the proportions of $CD8^{hi}TCR1^{-}$ T cell subsets present in the BAL and LN. Cells were isolated from the BAL and LN and stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD27, CD28 and CD25. Lymphocytes (R1) and $CD8^{hi}TCR1^{-}$ cells (red-gate R2) were gated as before and analysed for expression of surface molecules. Representative histograms are shown for an animal per group.

Compared to $CD8^{hi}TCR1^-$ T cells present in the LN, those present in the BAL display a decrease in the percentage expressing CD27 and an increase in the proportion of cells expressing IL-2R α chain CD25. Surprisingly, a similar percentage of $CD8^{hi}TCR1^-$T cells in the BAL and LN were found to express CD28, suggesting that although the $CD8^{hi}TCR1^-$ T cells present in the BAL display an activated phenotype they may not be terminally differentiated (Fig 4.2.3B and 4.2.5).

To further explore the changes with age (in blood) and location (in LN and BAL) in the proportions of $CD8^{hi}TCR1^-$T cell subsets, four-colour flow cytometry was used to analyse expression of CD45RO and one of the following surface molecules CD62L, CD45RA, CD27, CD28, CD3, CD2, CD5 and CD25 on $CD8^{hi}TCR1^-$ T cells.

Figure 4.2.6 shows that as the age of the animal increased, the frequency of the following $CD8^{hi}TCR1^-$T cell subsets present in blood increased: $CD45RO^+CD62L^-$, $CD45RO^+CD45RA^-$, $CD45RO^+CD27^+$, $CD45RO^+CD28^+$, $CD45RO^+CD28^-$, $CD45RO^+CD3^+$, $CD45RO^+CD2^+$, $CD45RO^+CD5^+$, $CD45RO^+CD25^+$ and $CD45RO^+CD25^-$. Compared with the two young groups, a greater percentage of $CD8^{hi}TCR1^-$T cells in older animals expressed the following phenotypes; $CD45RO^+CD45RA^+$, $CD45RO^+CD2^-$, $CD45RO^+CD5^-$ and $CD45RO^-CD27^-$.

Conversely, a decrease with age was observed in the frequency of the following $CD8^{hi}TCR1^-$T cell subsets: $CD45RO^-CD62L^+$, $CD45RO^-CD45RA^-$, $CD45RO^-CD27^+$, $CD45RO^-CD28^+$, $CD45RO^-CD3^+$, $CD45RO^-CD2^+$, $CD45RO^-CD5^+$, $CD45RO^-CD25^-$ (Fig.4.2.6 and 4.2.7). It was also observed that some subsets showed no significant change with age such as $CD8^{hi}TCR1^-CD45RO^+CD62L^+$ and $CD8^{hi}TCR1^-CD45RO^-CD28^-$ T cells.

It was found that the majority of resident $CD8^{hi}TCR1^-$T cells in LN expressed the following phenotypes: $CD45RO^-CD3^+$, $CD45RO^-CD62L^+$, $CD45RO^-CD45RA^+$, $CD45RO^-CD27^+$, $CD45RO^-CD28^+$ and $CD45RO^-CD25^-$ (Fig 4.2.8 and 4.2.9). Interestingly, the small population of memory $CD8^{hi}TCR1^-$T cells present in the LN can be further defined by expression of $CD45RO^+CD3^+$, $CD45RO^+CD62L^+$, $CD45RO^+CD45RA^{+/-}$, $CD45RO^+CD27^{+/-}$, $CD45RO^+CD28^{+/-}$ and $CD45RO^+CD25^-$ (Fig 4.2.8 and 4.2.9).

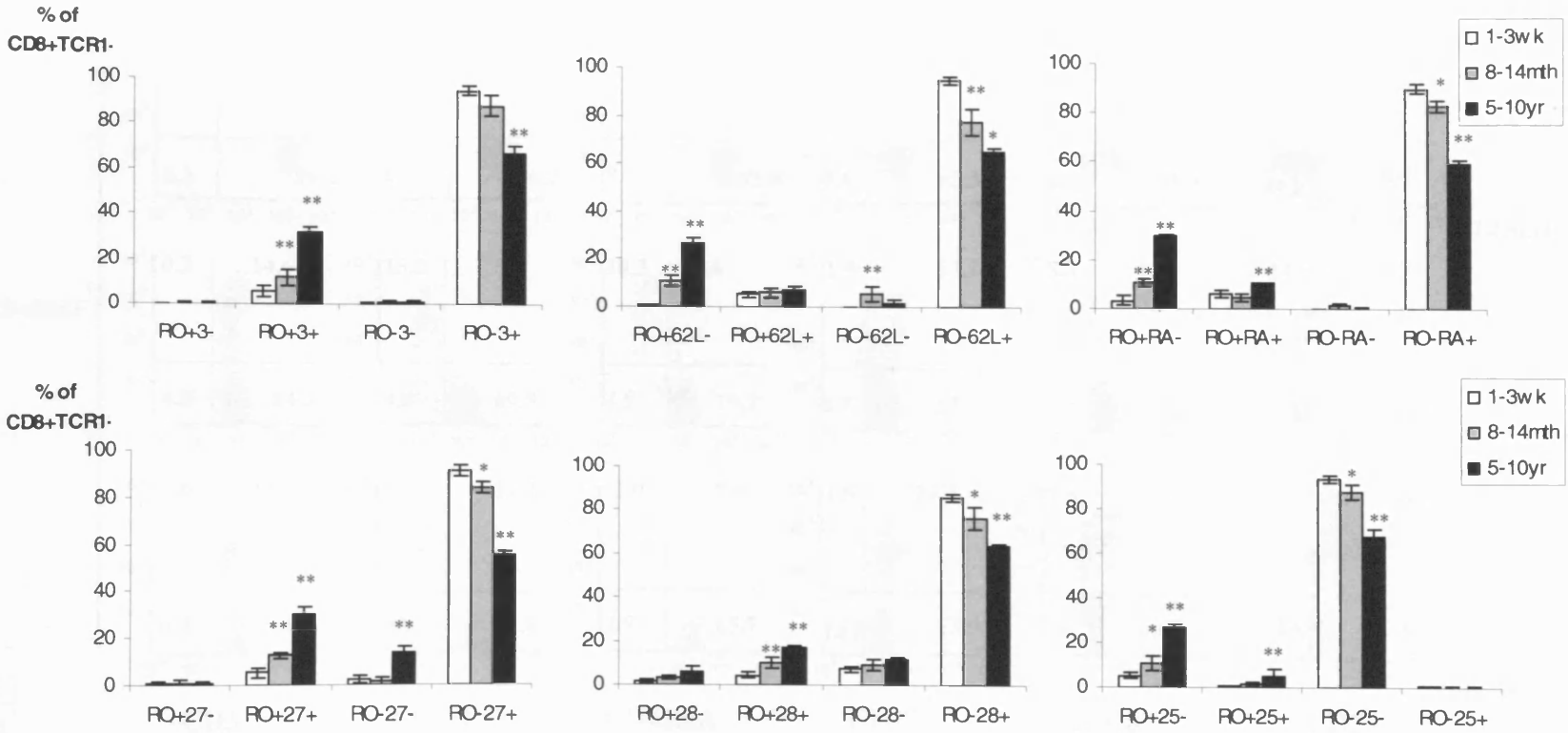


Figure 4.2.6 Age-related differences in the surface phenotype of $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cell subsets in blood. PBMC were analysed for expression of CD8, TCR1, CD45RO and expression of one of the following CD62L, CD45RA, CD27, CD28, CD3, and CD25 using 4-colour flow cytometry. PBMC were gated on $CD8^{+}TCR1^{-}$ T cells and the percentage of each subset was determined in animals from the three age groups (1-3wks, 8-14mth, 5-10yrs). The mean percentage and standard deviations of at least three animals per age group are shown. Differences between adjacent age groups that are statistically significant is shown on the older of the two groups as *$p<0.01$, **$p<0.001$.

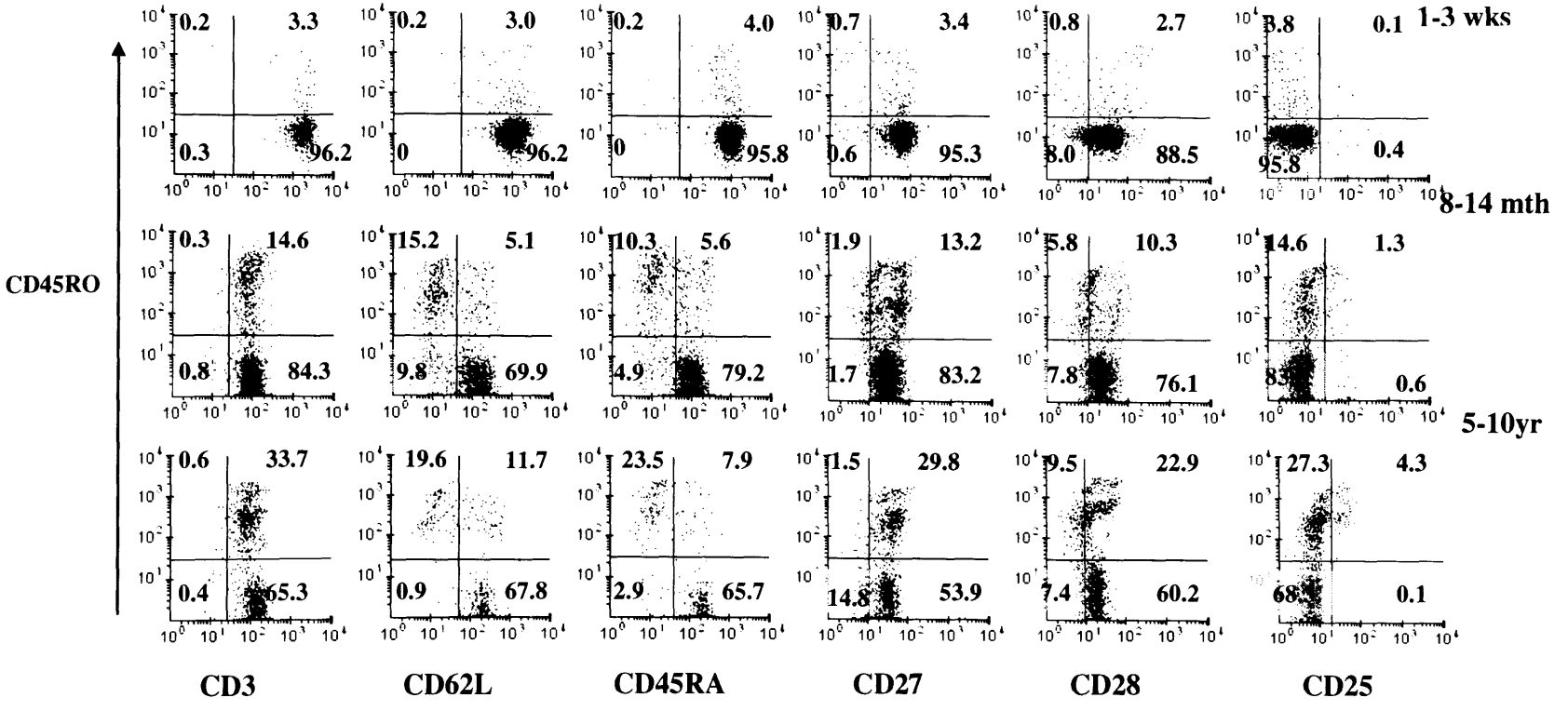


Figure 4.2.7 Dotplots illustrating differences in the expression of surface molecules on $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}$ $CD45RO^{-}$ T cell subsets with age. PBMC were isolated from blood and stained with antibodies to CD8, TCR1, CD45RO and one of the following CD3, CD62L, CD45RA, CD27, CD28 and CD25. PBMC was analysed using four-colour flow cytometry. Lymphocytes (R1) and $CD8^{hi}TCR1^{-}$ cells (R2) were gated as before and analysed for expression of surface molecules. Representative dotplots are shown for each age group.

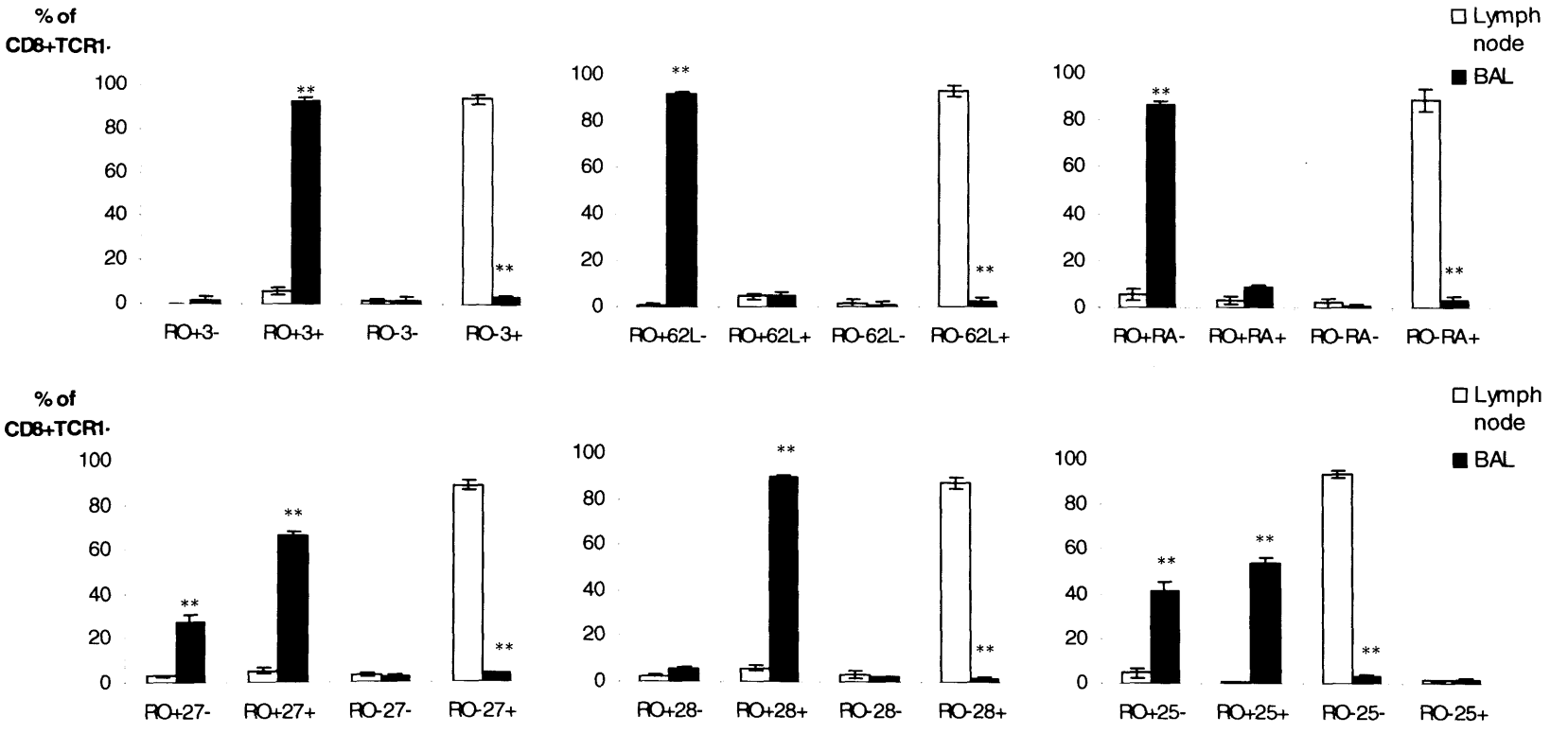


Figure 4.2.8 Differences in expression of surface molecules on $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cell subsets in the BAL and LN. Cells from the LN and BAL were analysed for expression of CD8, TCR1, CD45RO and one of the following surface molecules CD62L, CD45RA, CD27, CD28, CD3, and CD25. Cells were gated on $CD8^{hi}TCR1^{-}$ cells and the percentage of each subset was determined. The mean percentage and standard deviations of at least three animals per group are shown. Differences between the LN and BAL that are statistically significant is shown on the BAL as **$p<0.001$.

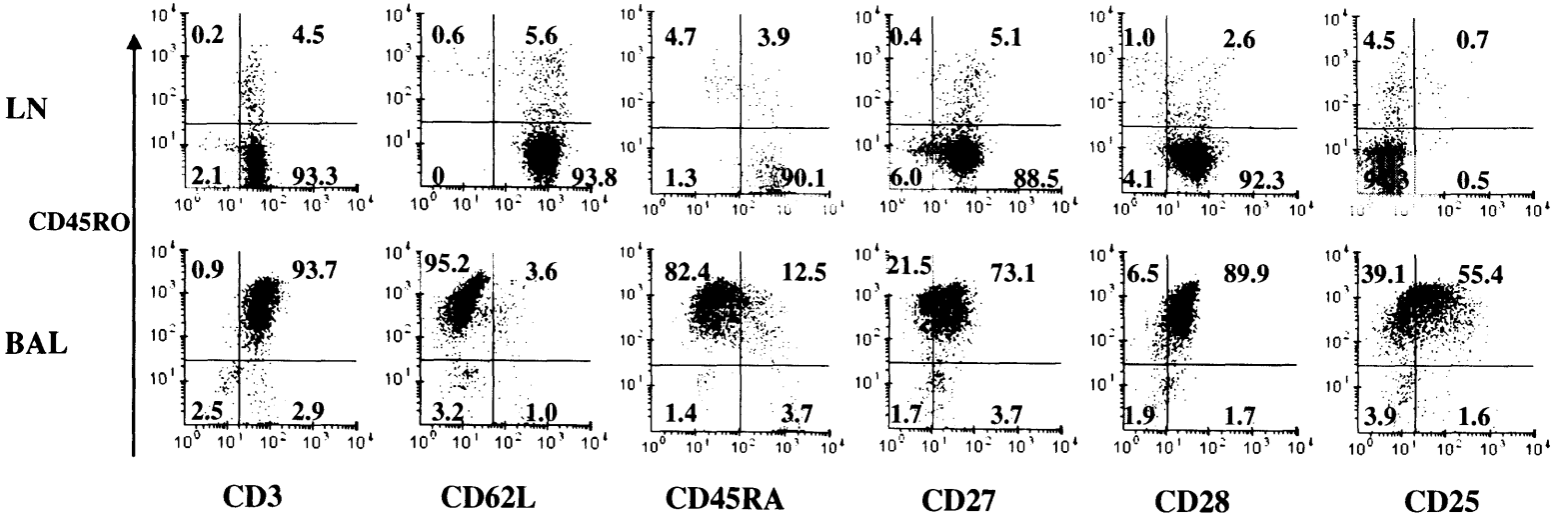


Figure 4.2.9 Dotplots illustrating differences in expression of surface molecules on $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cell subsets in BAL and LN. Cells were isolated from BAL and LN and analysed using flow cytometry for expression of CD8, TCR1, CD45RO and one of the following CD3, CD62L, CD45RA, CD27, CD28 and CD25. Lymphocytes (R1) and $CD8^{+}TCR1^{-}$ T cells (R2) were gated as before and analysed for expression of surface molecules. Representative plots are shown for each age group.

The predominant subsets of $CD8^{hi}TCR1^{-}$T cells present in the BAL were found to express the following phenotypes: $CD45RO^{+}CD3^{+}$, $CD45RO^{+}CD62L^{-}$, $CD45RO^{+}CD45RA^{-}$, $CD45RO^{+}CD27^{+/-}$, $CD45RO^{+}CD28^{+}$ and $CD45RO^{+}CD25^{+/-}$(Fig 4.2.8 and 4.2.9). However, smaller subsets of $CD8^{hi}TCR1^{-}$T cells were also found in the BAL that were $CD45RO^{+}CD62L^{+}$, $CD45RO^{+}CD45RA^{+}$ and $CD45RO^{+}CD28^{-}$ (Fig 4.2.8 and 4.2.9).

To investigate if age of the animal and anatomical location of the T cell has an effect on the expression of cell surface molecules by memory $CD8^{hi}TCR1^{-}$T cells defined by expression of CD45RO and naïve $CD8^{hi}TCR1^{-}$T cells by lack of CD45RO expression. This was achieved by gating on the $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells and analysing expression of CD62L, CD45RA, CD25, CD27, CD28 and CD3 by these two $CD8^{hi}TCR1^{-}$T cell subsets.

It was observed that the percentage of $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells expressing CD45RA and CD62L decreased and expression of CD25 increased with age (Fig 4.2.10A). Interestingly there is an increase with age in the frequency of $CD8^{hi}TCR1^{-}CD45RO^{-}CD27^{-}$ and $CD28^{-}$ T cells present in blood, this observation may be due to an increase in the number of primed $CD45RA^{+}$ T cells defined by lack of CD45RO, CD27 and CD28 and re-expression of CD45RA (Fig 4.2.10B).

The $CD8^{hi}TCR1^{-}CD45RO^{+}$T cells present in the BAL are predominantly $CD3^{+}$, $CD28^{+}$, $CD62L^{-}$, $CD45RA^{-}$, $CD27^{+}$ and $CD25^{+}$ (Fig.4.2.11A). In the BAL more than 95% of $CD8^{+}TCR1^{-}$ T cells express CD45RO, therefore the population of cells that lack CD45RO is extremely small. Figure 4.2.11B shows that a greater percentage of the $CD8^{hi}TCR1^{-}$ $CD45RO^{-}$ T cells in the BAL lack CD28 and CD27 compared to the $CD8^{hi}TCR1^{-}$ $CD45RO^{+}$T cells. In addition, the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells in the BAL are also mostly $CD45RA^{+}$, $CD62L^{+}$, $CD27^{+}$ and $CD25^{-}$ with a third expressing CD25 (Fig 4.2.11B).

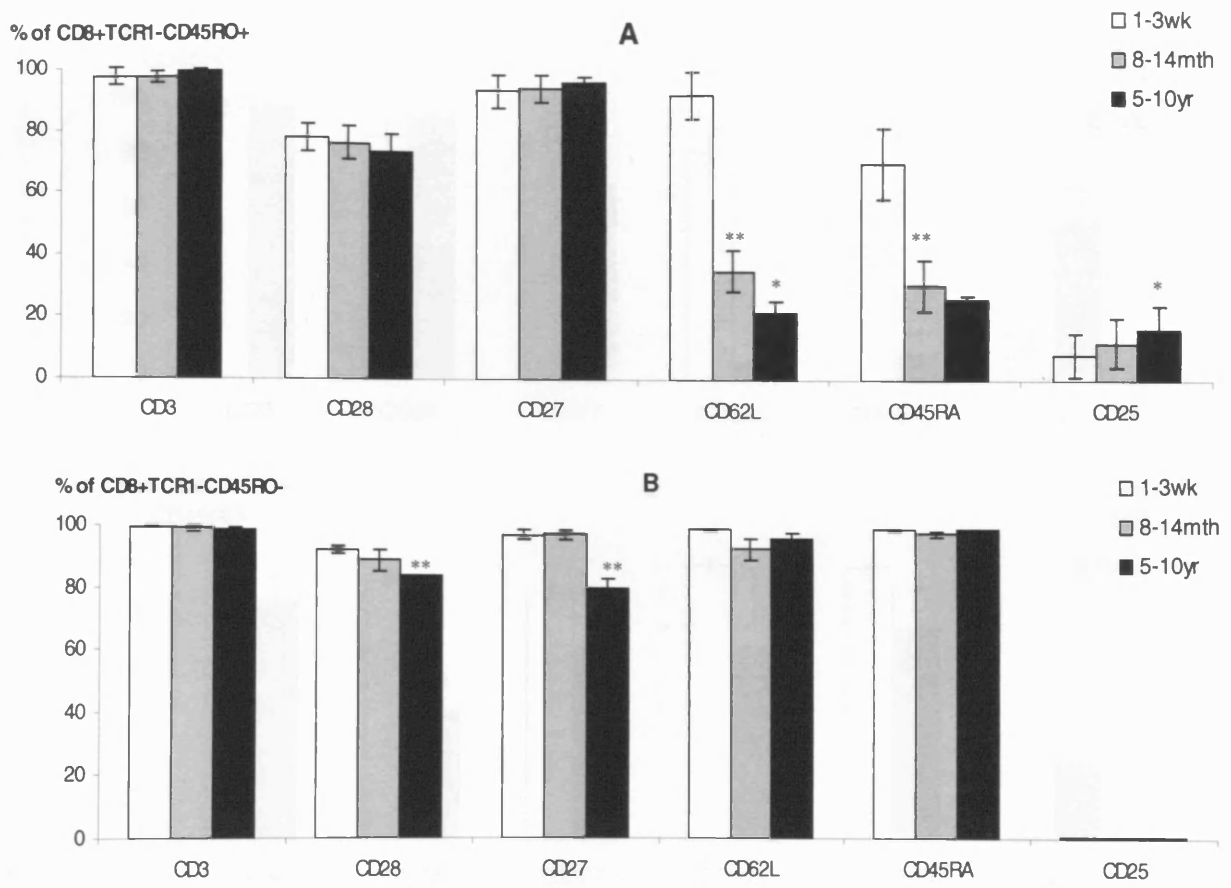


Figure 4.2.10 Surface phenotype of $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells present in blood. PBMC were analysed for expression of CD8, TCR1, CD45RO and one of the following CD62L, CD45RA, CD28, CD27, CD3 and CD25 using four-colour flow cytometry. PBMC was gated on either $CD8^{hi}TCR1^{-}$ $CD45RO^{+}$ or $CD8^{hi}TCR1^{-}$ $CD45RO^{-}$ cells and analysed for expression of one other surface molecule. The mean percentage and standard deviations of at least three animals per age group are shown. *$p<0.01$, **$p<0.001$.

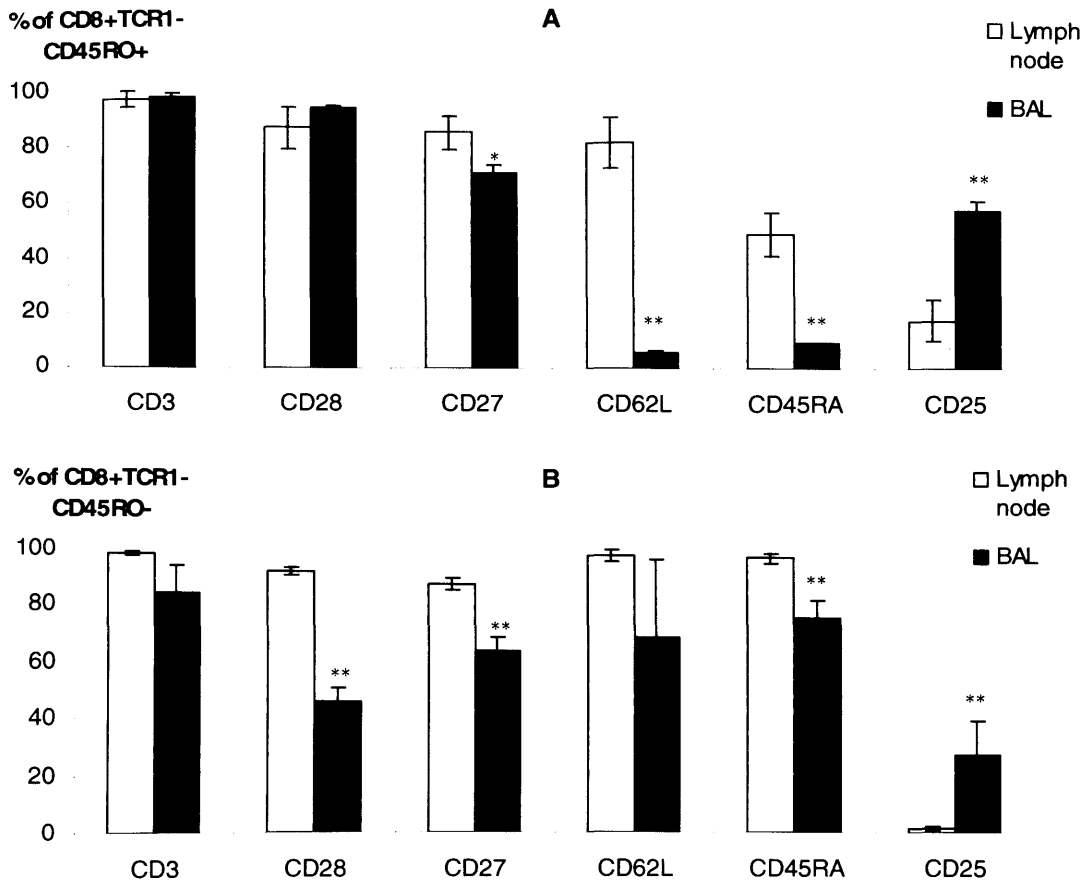


Figure 4.2.11 Surface phenotype of $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells in BAL and LN. Cells were analysed for expression of CD8, TCR1, CD45RO and one of the following CD62L, CD45RA, CD28, CD27, CD3 and CD25 using four-colour flow cytometry. Gates were placed around the $CD8^{hi}TCR1^{-}$ $CD45RO^{+}$ (A) and $CD8^{hi}TCR1^{-}$ $CD45RO^{-}$ (B) and expression of one other surface molecule was assessed on these two populations. The mean percentage and standard deviations of at least three animals per age group are shown. *p<0.01, **p<0.001.

The expression of surface molecules on some of the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells in the BAL suggests that although these cells were termed naïve because they lack CD45RO some may be effector cells. It is possible that these effector cells have yet to express CD45RO or are a population of repetitively stimulated memory T cells that have lost CD45RO and re-expressed CD45RA or perhaps a combination of the two.

The small population of memory $CD8^{hi}TCR1^{-}$T cells present in LN are shown in figure 4.2.11A to be mostly; $CD3^{+}$, $CD62L^{+}$, $CD28^{+}$, $CD27^{+}$, $CD45RA^{-}$ and $CD25^{-}$. The population of $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells in the LN were predominantly: $CD3^{+}$, $CD62L^{+}$, $CD45RA^{+}$, $CD27^{+}$, $CD28^{+}$ and $CD25^{-}$ (Fig 4.2.11B). This population of naïve T cells in the LN, unlike the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells present in the BAL that resemble a population of primed T cells, more resemble a population of naïve $CD8^{hi}TCR1^{-}$T cells.
Differences were observed in the proportion of each subset of $CD8^{hi}TCR1^{-}$T cells ($CD45RO^{+}$ and $CD45RO^{-}$) that express certain surface molecules. Comparisons of the these subsets found at two different locations (i.e. the BAL and LN) shows that $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells present in LN express a different pattern of surface molecules compared to $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells resident in the BAL and the same is true for the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells found at the two different sites.
A greater percentage of $CD8^{hi}TCR1^{-}CD45RO^{+}$T cells in the LN express CD62L and CD27 compared to $CD8^{hi}TCR1^{-}CD45RO^{+}$T cells in the BAL. A greater proportion of $CD8^{hi}TCR1^{-}CD45O^{-}$ T cells in the BAL were found to be $CD25^{+}$, $CD28^{-}$, $CD27^{-}$ and $CD62L^{-}$ compared to $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells present in LN.

4.3 Expression of perforin by $CD8^{hi}TCR1^{-}$T cells in blood, LN and BAL

Acquisition of effector functions occurs after exposure of a naïve T cell to its antigen. The antigen must be presented together with the optimal level of co-stimulation by an antigen presenting cell (APC), to result in the clonal expansion and differentiation of the naïve T cell into effector T cells and potentially into memory T cells (Agarwal and Rao 1998; Bachmann, Barner et al. 1999; Veiga-Fernandes, Walter et al. 2000). Perforin functions by forming pores in the membrane of target cells and is contained inside cytotoxic granules.

Perforin is thought to aid the release of other cytotoxic mediators contained within granules which include granzymes and granulysin into the target cell (Pinkoski, Hobman et al. 1998; Stenger, Hanson et al. 1998; Metkar, Wang et al. 2002). Expression of perforin at the mRNA and protein level is only induced in activated/effector/memory T cells (Kelso, Costelloe et al. 2002).

To investigate whether the age-dependent increase in T cells expressing an effector/memory surface phenotype is reflected in an increase in the percentage of cells expressing the cytolytic molecule perforin. The percentage of $CD8^{hi}TCR1^{-}$T cells that express perforin was analysed in PBMC from animals of different ages and cells from the LN and BAL.

It was observed that the percentage of $CD8^{hi}TCR1^{-}$T cells that express perforin increases in blood with age (Fig 4.3.1 and 4.3.2). It is demonstrated in figures 4.3.1 and 4.3.2 that a higher percentage of $CD8^{hi}TCR1^{-}$T cells in the BAL express perforin compared to LN (Fig 4.3.1 and 4.3.2).

The next objective was to identify which subsets of $CD8^{hi}TCR1^{-}$T cells in cattle express perforin. Four-colour flow cytometry was used to analyse expression of surface molecules and intracellular perforin by $CD8^{hi}TCR1^{-}$ T cells in PBMC and in LN and BAL.

Figure 4.3.3 and 4.3.4 shows that an increase in the percentage of perforin expressing $CD8^{hi}TCR1^{-}$ T cells with age in blood can be found within the following subsets: $CD62L^{-}$, $CD45RA^{-}$, $CD25^{-}$, $CD28^{-}$, $CD45RO^{+}$, $CD27^{+}$, $CD28^{+}$ and $CD25^{-}$. Smaller increases were observed in the frequency of perforin expressing $CD8^{hi}TCR1^{-}$T cell that are; $CD62L^{+}$, $CD45RA^{+}$, $CD28^{+}$, $CD25^{+}$ and $CD45RO^{-}$ (Fig 4.3.3 and 4.3.4). Interestingly an increase in the percentage of $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells that lack expression of perforin was observed, it is unclear what the effector function of these cells are (Fig 4.3.3 and 4.3.4).

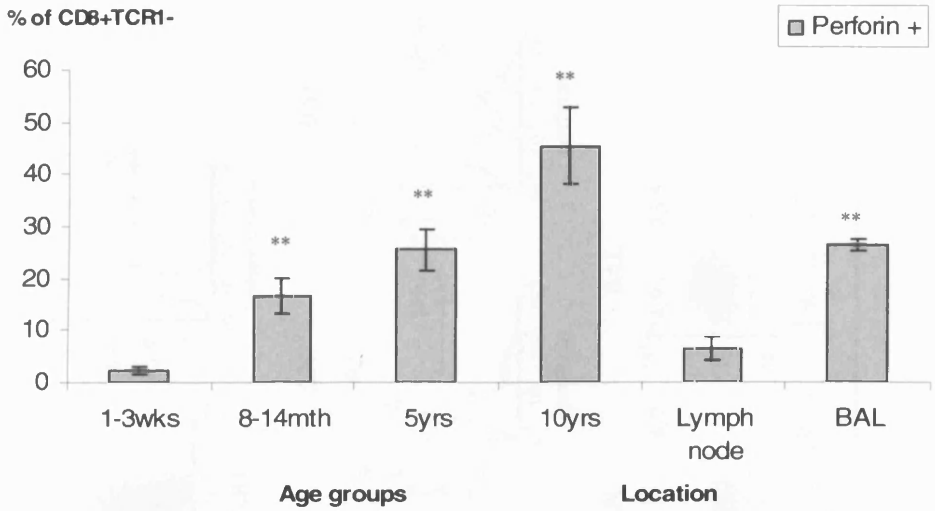


Figure 4.3.1 Comparison of the percentage of $CD8^{hi}TCR1^{-}perforin^{+}$ T cells in blood, BAL and LN. Cells were stained with antibodies to CD8 and TCR1 on cell surface. Cells were then fixed, permeabilised and stained with anti-human perforin mAb which cross-reacts with bovine perforin. The mean and standard deviation are shown for each group. Each group is comprised of at least three animals. Differences between adjacent age groups or between the LN and BAL that are statistically significant is shown on the older of the two groups or on the BAL as **$p<0.001$.

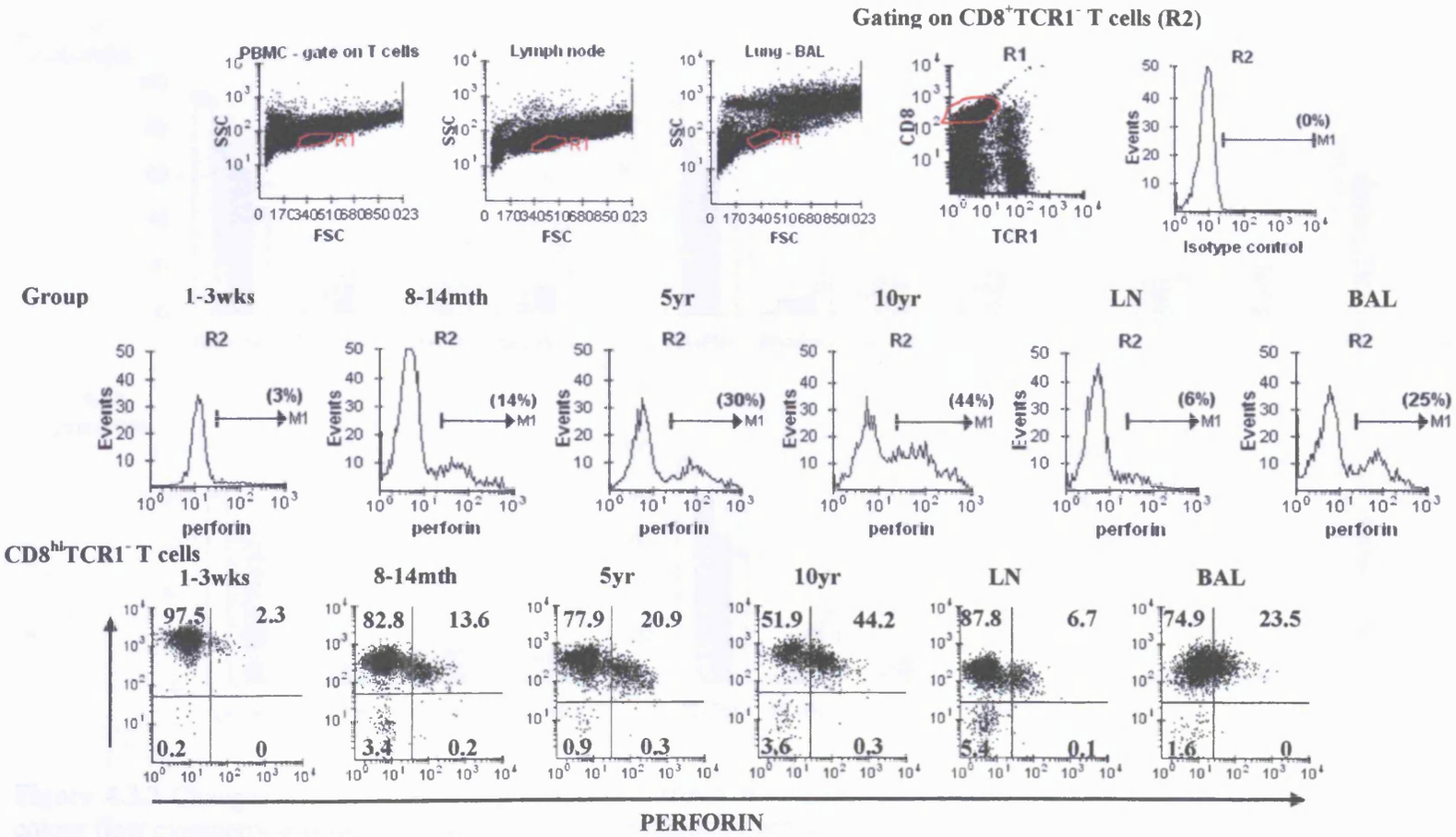


Figure 4.3.2 Differences in the frequency of perforin expressing $CD8^{hi}TCR1^-$ T cells in blood with age and between $CD8^{hi}TCR1^-$ T cells resident in LN and BAL. PBMC and cells from LN and BAL were stained with antibodies to CD8 and γδ T cell receptor (TCR1), before being fixed, permeabilised and stained with antibody to perforin. Lymphocytes (R1) and $CD8^+TCR1^-$ T cells (R2) were gated as before and analysed for expression of perforin. Representative dotplots are shown from one animal from each group.

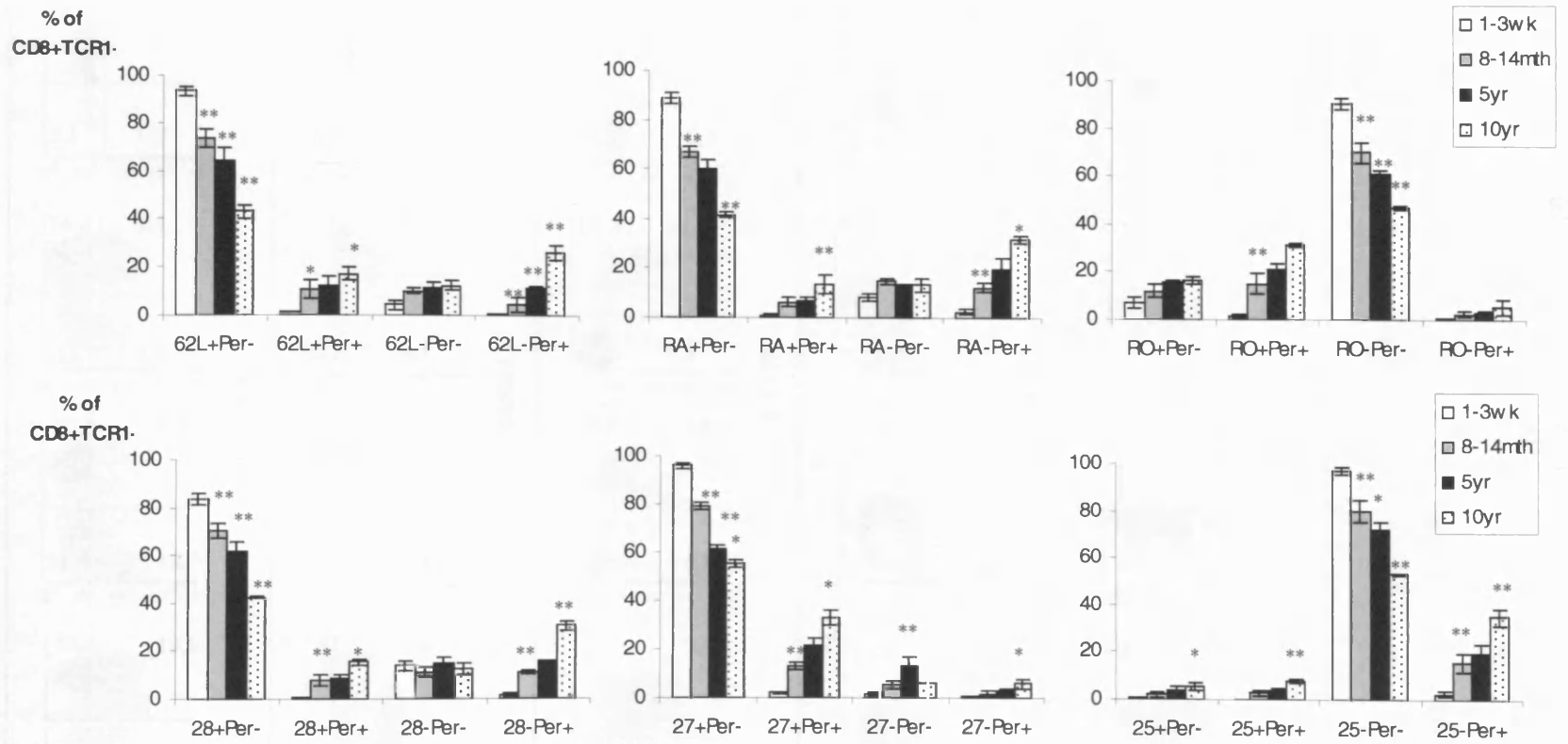


Figure 4.3.3 Changes with age in the expression of perforin and surface molecules on $CD8^{hi}TCR1^{-}$ T cell subsets in blood. Four-colour flow cytometry was used to investigate the expression of perforin by $CD8^{hi}TCR1^{-}$ T cells. PBMC were stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD27, CD28 and CD25. Cells were then fixed, permeabilised and stained with an antibody to human perforin. The mean percentage and standard deviations of at least three animals per age group are shown. Differences between adjacent age groups that are statistically significant is shown on the older of the two groups as *$p<0.01$, **$p<0.001$.

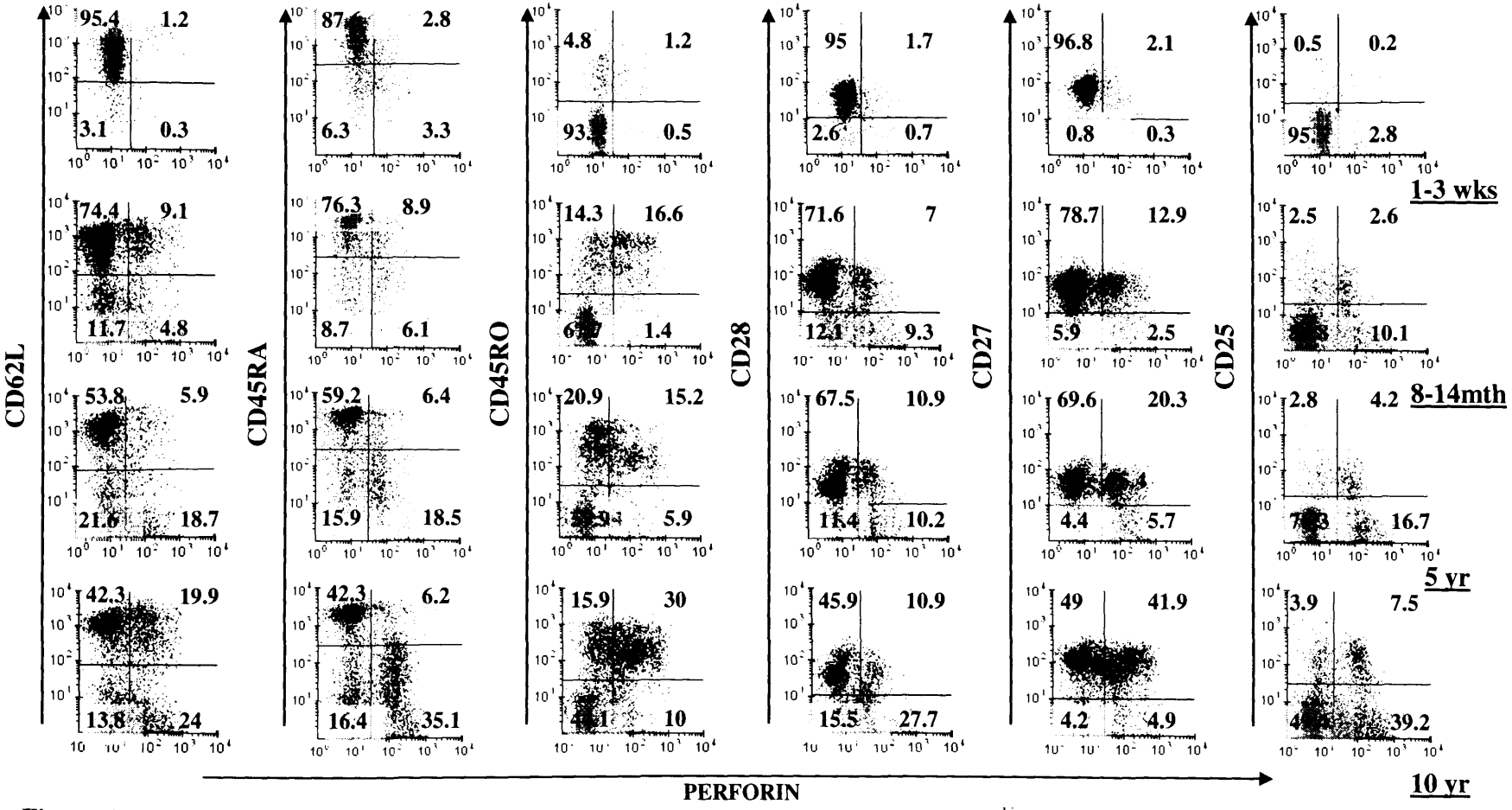


Figure 4.3.4 Dotplots illustrating age-related differences in the expression of perforin by $CD8^{hi}TCR1^{-}$ T cell subsets in blood. Four-colour flow cytometry was used to analyse expression of perforin and surface molecules on CD8hiTCR1- T cells. PBMC was isolated from animals of different ages and stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD27, CD28 and CD25. Cells were then fixed, permeabilised and stained with an antibody to perforin. Lymphocytes (R1) and $CD8^{hi}TCR1^{-}$ cells (R2) were gated as before. Representative dotplots are shown for one animal from each age group.

The results in figures 4.3.5 and 4.3.6 displaying expression of surface molecules on $CD8^{hi}TCR1^{-}$T cells present in the BAL is preliminary data because the four-colour flow cytometry used to exclude the γδ T cells was only performed once due to availability of tissues. However, three-colour analysis of perforin and surface molecule expression on $CD8^{hi}$ cells present in BAL showed similar results as the population of $CD8^{hi}TCR1^{+}Perforin^{+}$ T cells in the BAL is relatively small.

Figure 4.3.5 shows that the $CD8^{hi}TCR1^{-}Perforin^{+}$ T cells in the BAL are found within different subsets compared to the LN. The $CD8^{hi}TCR1^{-}Perforin^{+}$ T cells present in the BAL were found predominantly within the following subsets: $CD62L^{-}$, $CD45RA^{-}$, $CD45RO^{+}$, $CD28^{+}$, $CD27^{+/-}$ and $CD25^{+/-}$(Fig 4.3.5). The small population of perforin expressing $CD8^{hi}TCR1^{-}$T cell resident in the LNs were found within the $CD62L^{+/-}$, $CD45RA^{+/-}$, $CD28^{-}$, $CD27^{+}$, $CD45RO^{+}$ and $CD25^{-}$ subsets (Fig 4.3.5).

After determining which subpopulations of $CD8^{hi}TCR1^{-}$T cells contain the perforin expressing cells, by gating on the $CD8^{hi}TCR1^{-}Perforin^{+}$ T cells, expression of the cell surface molecules was analysed directly on these cells.

The majority of $CD8^{hi}TCR1^{-}Perforin^{+}$ T cells in blood expressed the phenotype: $CD3^{+}$, $CD45RA^{-}$, $CD28^{-}$, $CD62L^{+/-}$, $CD27^{+}$, $CD45RO^{+}$ and $CD25^{-}$ (Fig 4.3.7A). The percentage of $CD8^{hi}TCR1^{-}Perforin^{+}CD25^{+}$ T cells in blood increased with age and $CD8^{hi}TCR1^{-}Perforin^{+}CD62L^{+}$ decreased with age (Fig 4.3.7A).

The $CD8^{hi}TCR1^{-}Perforin^{+}$ T cells present in the BAL were found to be mostly $CD3^{+}$, $CD62L^{-}$, $CD45RA^{-}$, $CD45RO^{+}$, $CD25^{+/-}$, $CD28^{+}$ and $CD27^{+}$(Fig 4.3.7B). Whereas the $CD8^{hi}TCR1^{-}Perforin^{+}$ T cells resident in the LN are predominantly $CD3^{+}$, $CD62L^{+}$, $CD45RA^{+/-}$, $CD28^{-}$, $CD25^{-}$, $CD45RO^{+}$ and $CD27^{+}$(Fig 4.3.7B).

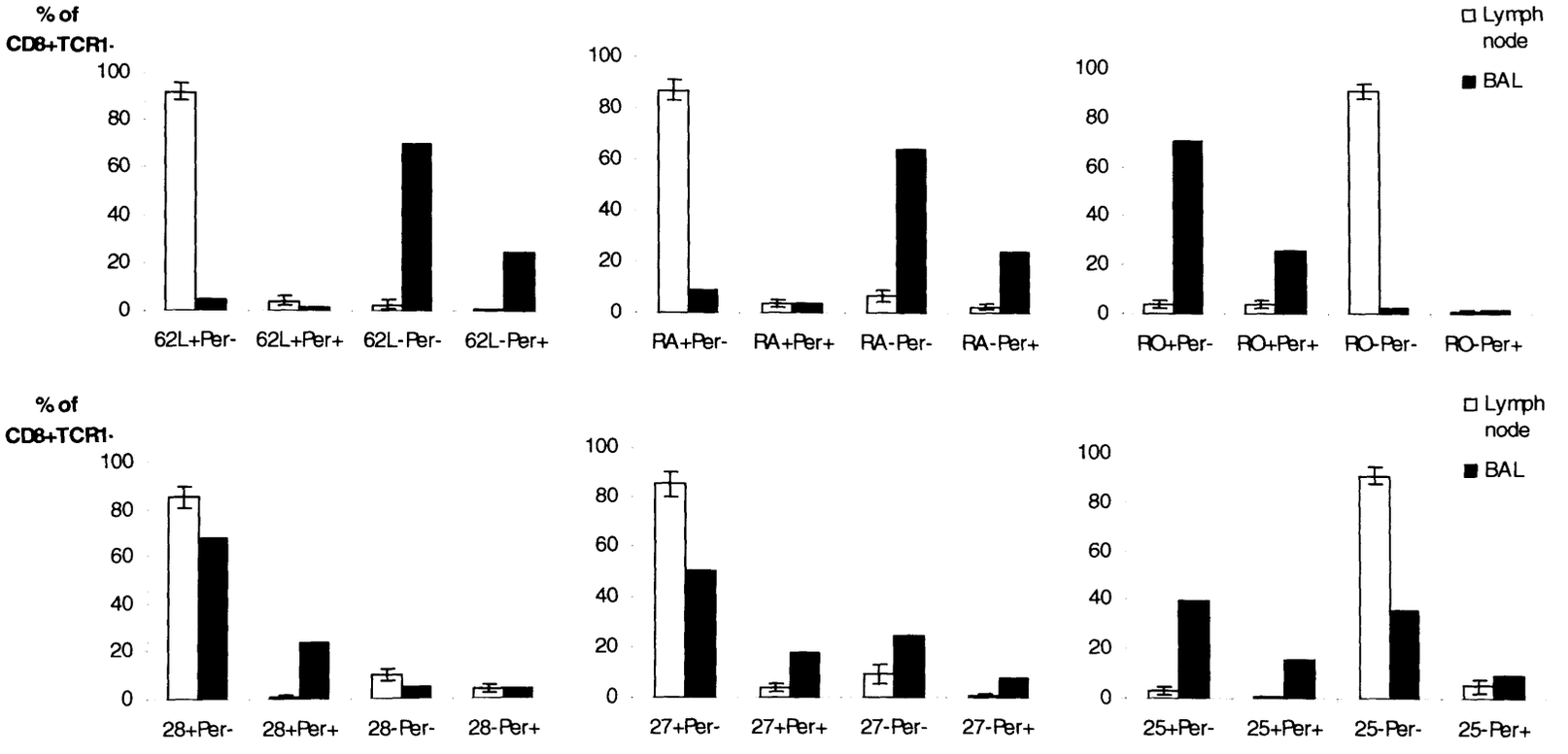


Figure 4.3.5 Expression of perforin in $CD8^{hi}TCR1^{-}$ T cell subsets from BAL and LN. Four-colour flow cytometry was used to investigate the expression of perforin by $CD8^{hi}TCR1^{-}$ T cell subsets present in BAL and LN. Cells were isolated from the LN and BAL were stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD27, CD28 and CD25. Cells were then fixed, permeabilised and stained with an antibody to human perforin. The mean percentage and standard deviations of at least three animals per age group are shown.

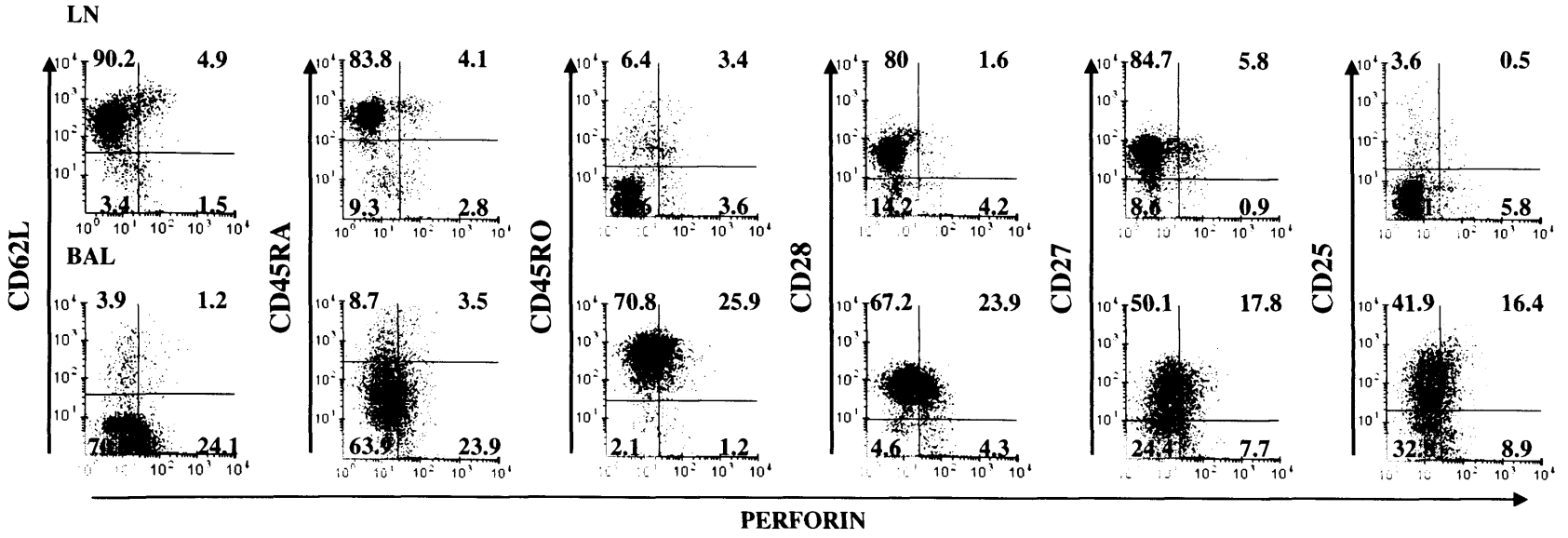


Figure 4.3.6 Dotplots showing the surface phenotype of IFN-γ expressing $CD8^{hi}TCR1^-$ T cell subsets present in BAL and LN. Cells isolated from the LN and BAL were stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD27, CD28 and CD25. Cells were then fixed, permeabilised and stained with an antibody to human perforin. Lymphocytes (R1) and $CD8^{hi}TCR1^-$ cells (R2) were gated as before and analysed for expression of surface molecules and perforin. Representative dotplots are shown from each group.

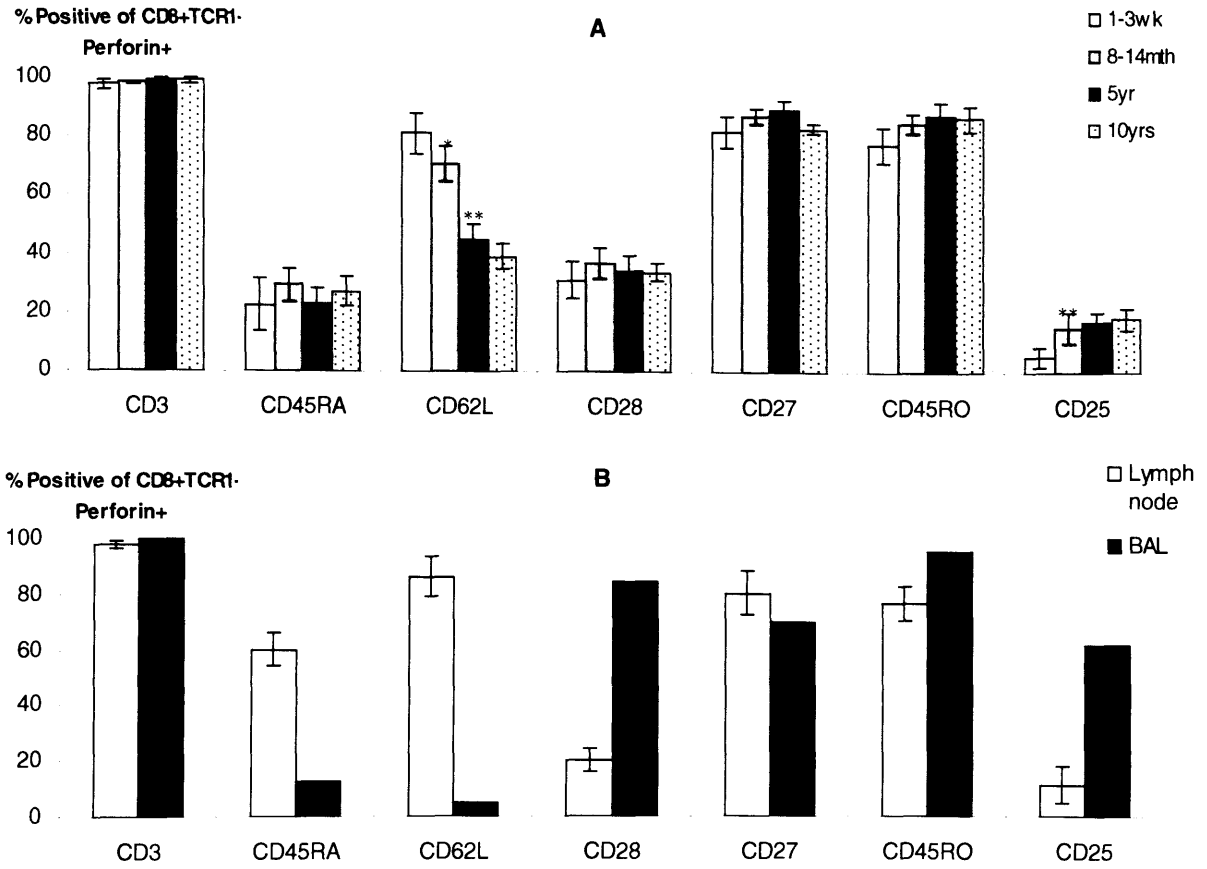


Figure 4.3.7 Surface phenotype of $CD8^{hi}TCR1^{-}perforin^{+}$ T cells present in blood from animals of different ages (A) and in LN and BAL (B). PBMC and cells from the LN and BAL were analysed for expression of CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD28, CD27, CD3 and CD25 using four-colour flow cytometry. Cells were then fixed, permeabilised and stained with an antibody to perforin. The $CD8^{hi}TCR1^{-}$ $Perforin^{+}$ T cells were gated and analysed for expression of surface molecules. The mean percentage and standard deviations of at least three animals per age group are shown. Differences between adjacent age groups that are statistically significant is shown on the older of the two groups as * $p<0.01$, ** $p<0.001$.

4.4 Expression of IFN-γ following mitogen stimulation of $CD8^{hi}TCR1^{-}$T cells in blood, LN and BAL.

An important effector function of effector $CD8^{+}$ T cells, in particular in TB infection, is the production of IFN-γ, which is thought to activate macrophages to kill intracellular pathogens. In order to further define different subsets of $CD8^{hi}TCR1^{-}$T cells in cattle, PBMC and cells from LN and BAL were cultured for 5 hrs with mitogen PMA/I/BFA or BFA alone. This short stimulation period is thought to activate only effector or memory T cells to produce IFN-γ as this period of time is not adequate to induce naïve T cells to differentiate into effector cells. Cells were then stained with antibodies to CD8 and TCR1 before being fixed, permeabilised and stained with an antibody to bovine IFN-γ.

The percentage of $CD8^{hi}TCR1^{-}$T cells present in blood that have the ability to express IFN-γ increased with age (Fig 4.4.1). A greater percentage of $CD8^{hi}TCR1^{-}$T cells in the BAL expressed IFN-γ compared to the LNs (Fig 4.4.1 and 4.4.2).

To define which $CD8^{hi}TCR1^{-}$T cell subsets expressed IFN-γ, cells were stimulated as described and analysed for expression of surface molecules together with IFN-γ.

In blood, IFN-γ^{+} cells increased with age among the $CD8^{hi}TCR1^{-}$T cells that are $CD62L^{-}$, $CD45RA^{-}$, $CD45RO^{+}$, $CD28^{+}$, $CD25^{-}$ and $CD27^{+/-}$ was observed (Fig 4.4.3 and 4.4.4). The IFN-γ expressing $CD8^{hi}TCR1^{-}$T cells present in LN were found to be $CD62L^{+}$, $CD45RA^{+}$, $CD28^{+}$, $CD27^{+}$ and CD25- (Fig 4.4.5 and 4.4.6). In contrast to LN, the IFN-$\gamma^{+}CD8^{hi}TCR1^{-}$ T cells present in the BAL were $CD62L^{-}$, $CD45RA^{-}$, $CD45RO^{+}$, $CD28^{+}$, $CD27^{+/-}$ and $CD25^{+/-}$ (Fig 4.4.5 and 4.4.6).

The previous results determined which $CD8^{hi}TCR1^{-}$T subsets expressed IFN-γ, by gating on the $CD8^{hi}TCR1^{-}$IFN-γ^{+} T cells it was possible to directly analyse expression of surface molecules on these cells.

Figure 4.4.7A shows that the percentage of $CD8^{hi}TCR1^{-}$IFN-γ^{+}T cell present in blood that express CD45RA, CD27 and CD45RO decreased with age and the percentage expressing CD25 increased with age.

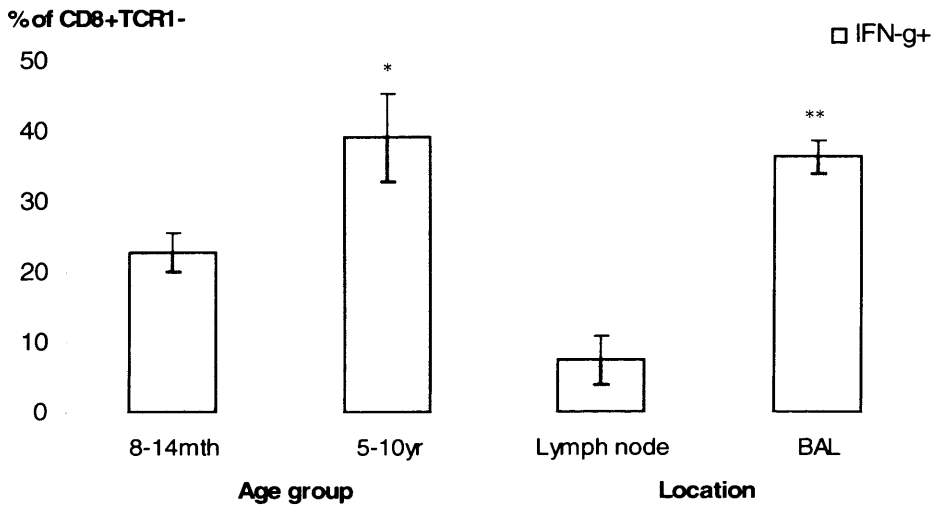


Figure 4.4.1 Comparison of the percentage of IFN-γ expressing $CD8^{hi}TCR1^-$ T cells from blood, BAL and LN. Cells were stimulated for 5hrs with PMA/I/BFA or BFA alone Flow cytometry was used to stain cells from PBMC, BAL and LN with antibodies to CD8 and TCR1. Cells were then fixed, permeabilised and stained with an antibody to bovine IFN-γ. $CD8^+TCR1^-$ T cells were gated and analysed for expression of IFN-γ. The mean and standard deviation are shown for each group. Each group is comprised of at least three animals. Differences between adjacent age groups or between the LN and BAL that are statistically significant is shown on the older of the two groups or on the BAL as * $p<0.01$, ** $p<0.001$.

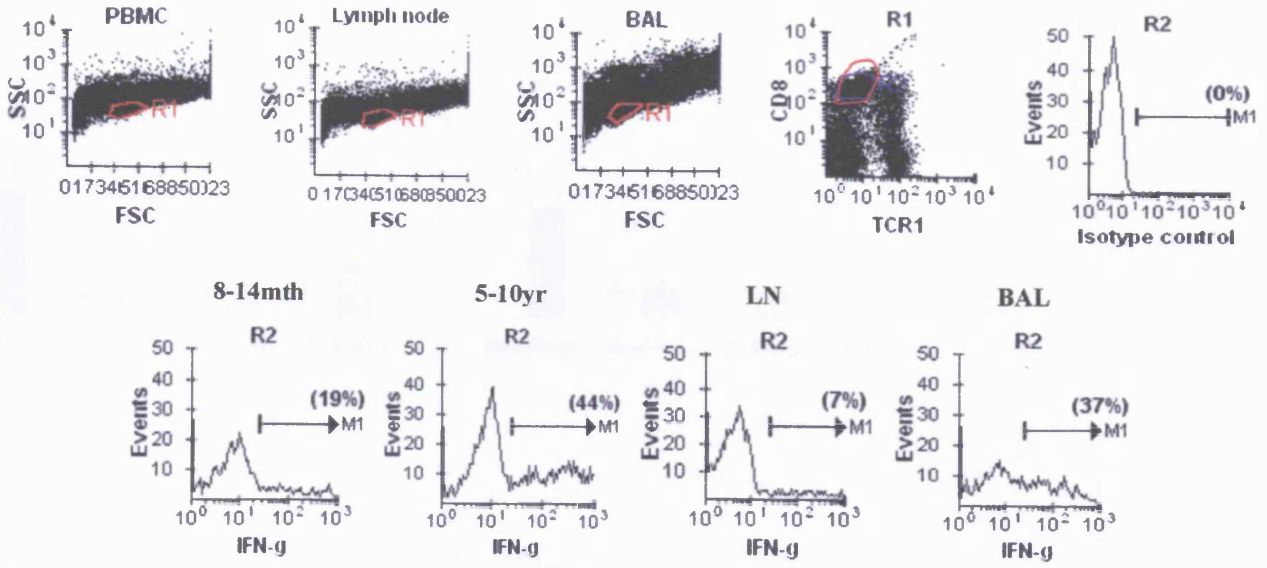


Figure 4.4.2 Dotplots showing expression of IFN-γ by $CD8^{hi}TCR1^{-}$ T cells present in the blood, BAL and LN. Flow cytometry was used to stain cells from PBMC, BAL and LN with antibodies to CD8 and TCR1. Cells were then fixed, permeabilised and stained with an antibody to bovine IFN-γ. Lymphocytes (R1) and $CD8^{hi}TCR1^{-}$ cells (R2) were gated as before and analysed for expression of IFN-γ. Representative dotplots and histograms are shown for each group.

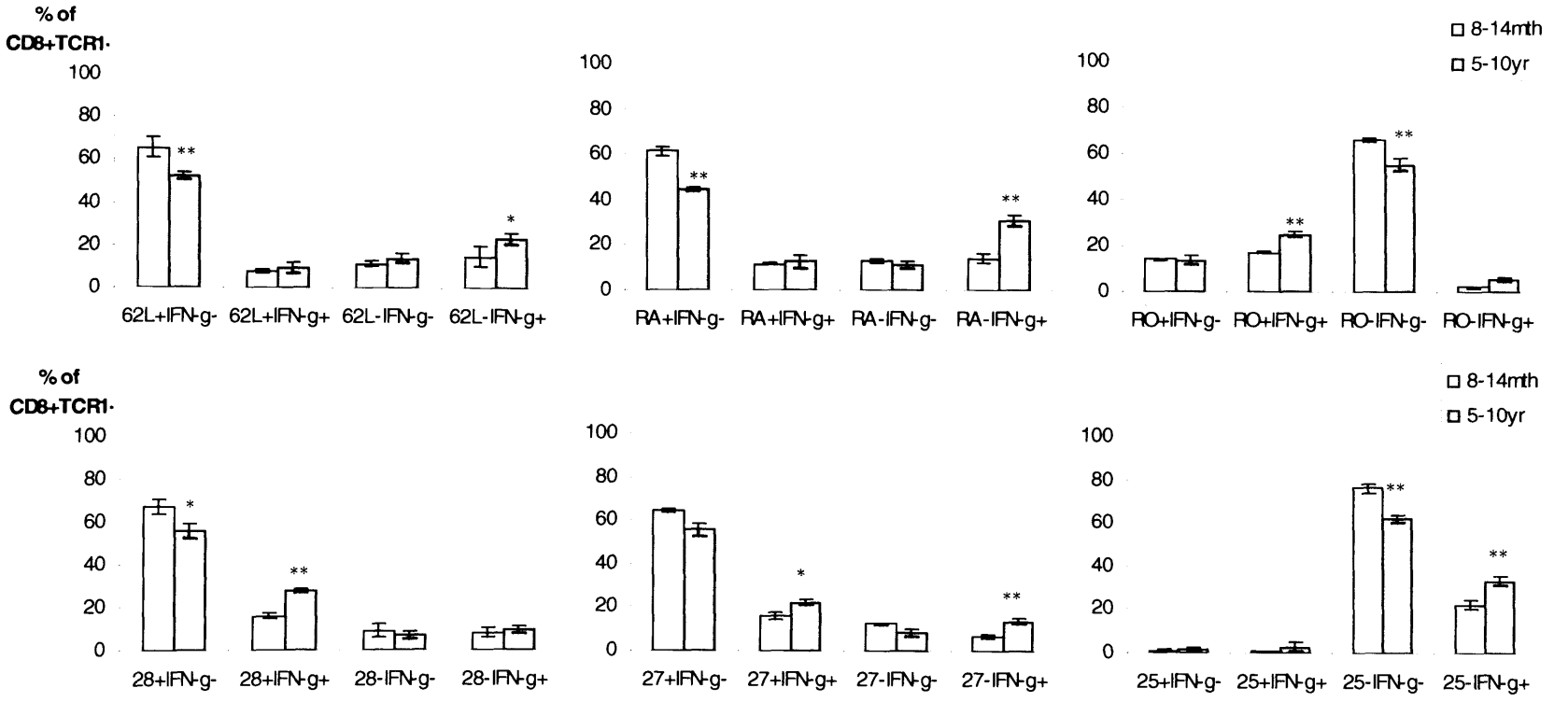


Figure 4.4.3 Age-associated differences in the frequency of IFN-γ expressing $CD8^{hi}TCR1^{-}$ T cell subsets in blood. PBMC was isolated from animals in the two age groups and stimulated for 5hrs with PMA/I/BFA or BFA alone. Cells were then stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD28, CD27 and CD25 before being fixed, permeabilised and stained with an antibody which recognises bovine IFN-γ. Gates were placed around the lymphocyte fraction and $CD8^{hi}TCR1^{-}$ T cells and the expression of surface molecules and IFN-γ was investigated. The mean and standard deviation are shown for at least three animals per group. Differences between adjacent age groups that are statistically significant is shown on the older of the two groups as * $p<0.01$, ** $p<0.001$.

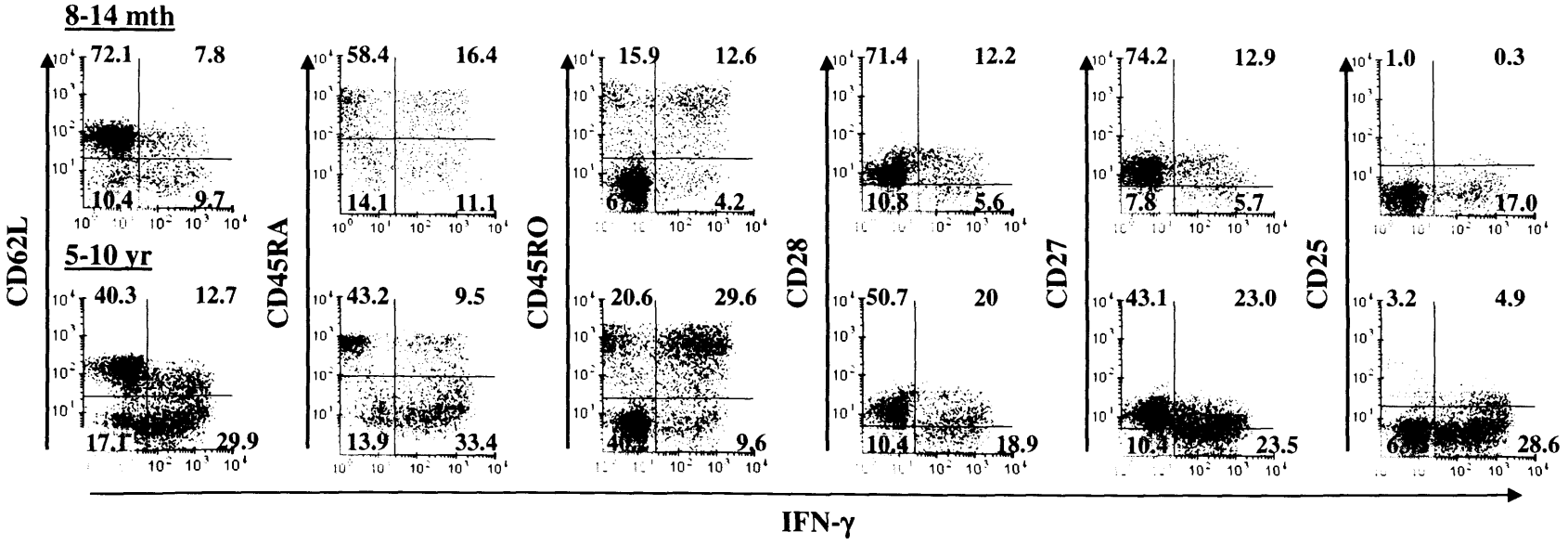


Figure 4.4.4 Dotplots showing expression of IFN-γ by $CD8^{hi}TCR1^{-}$ T cell subsets present in the blood. PBMC were stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD28, CD27 and CD25 before being fixed, permeabilised and stained with an antibody which recognises bovine IFN-γ. Lymphocytes (R1) and $CD8^{hi}TCR1^{-}$ cells (R2) were gated as before and were analysed for expression of surface molecules and IFN-γ. Representative dotplots are shown for each group.

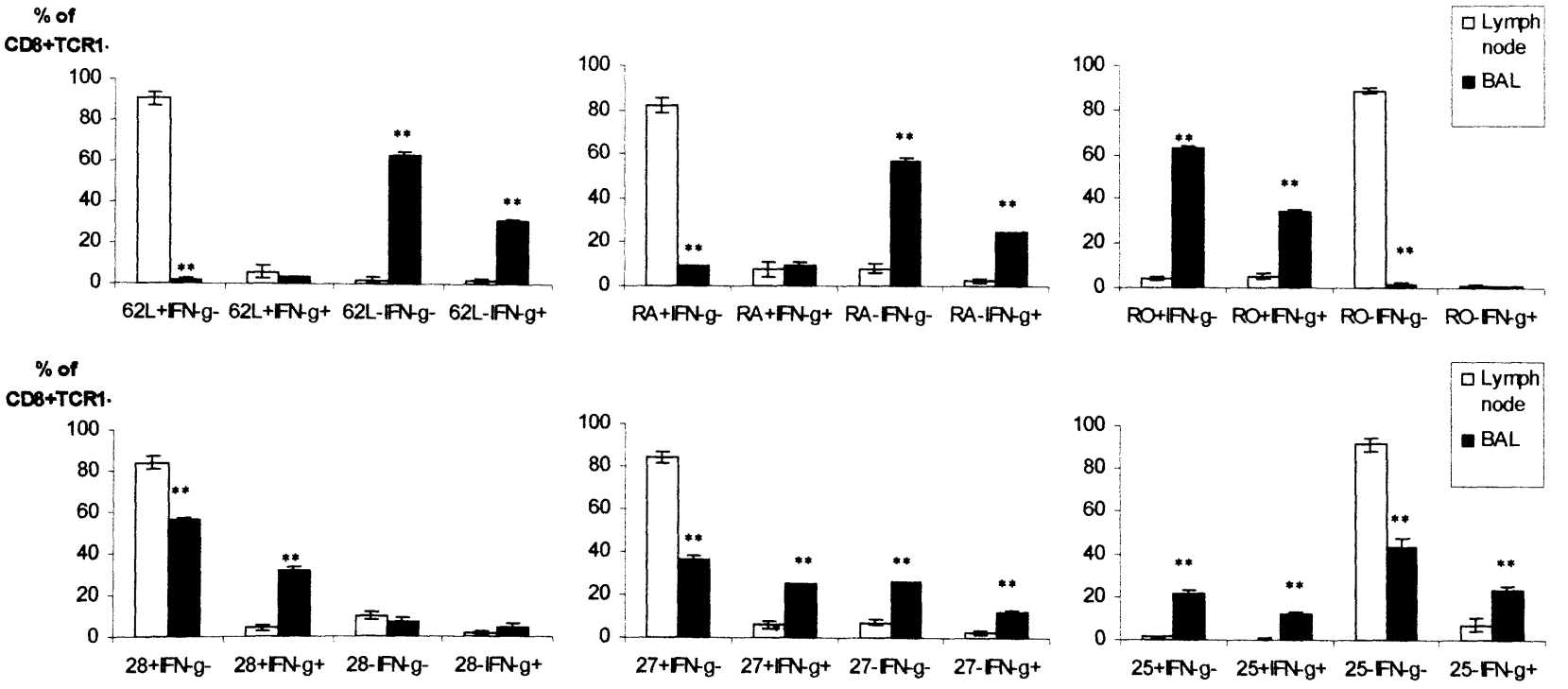


Figure 4.4.5 Differences in the expression of IFN-γ by $CD8^{hi}TCR1^{-}$ T cells subsets present in LN and BAL. Cells isolated from LN and BAL were stimulated for 5hrs with PMA/I/BFA or BFA alone. Cells were then stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD28, CD27 and CD25 before being fixed, permeabilised and stained with an antibody which recognises bovine IFN-γ. Gates were placed around the $CD8^{hi}TCR1^{-}$ T cells and the expression of surface molecules and IFN-γ was investigated. The mean and standard deviation are shown for at least three animals per group. Differences between the LN and BAL that are statistically significant is shown on the BAL as * $p<0.01$, ** $p<0.001$.

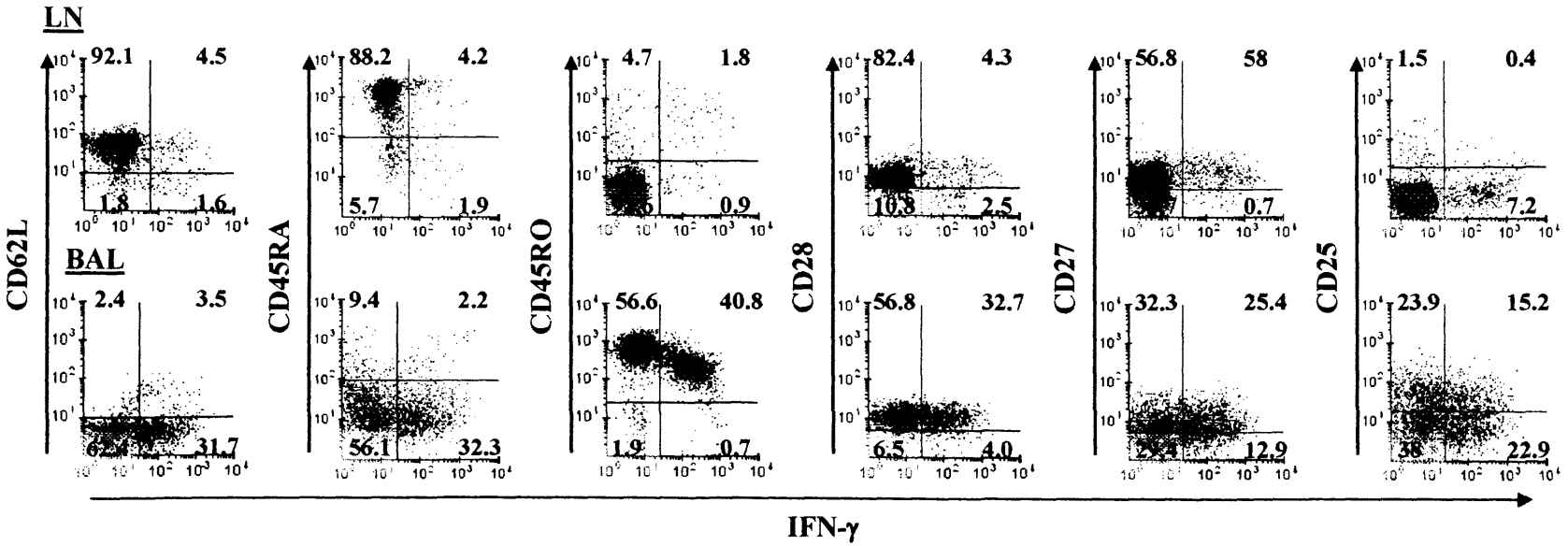


Figure 4.4.6 Dotplots showing expression of IFN-γ and surface molecules by $CD8^{hi}TCR1^{-}$ T cells present in the BAL and LN. Cells isolated from the LN and BAL were stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD27, CD28 and CD25. Cells were then fixed, permeabilised and stained with an antibody to bovine IFN-γ. Lymphocytes (R1) and $CD8^{hi}TCR1^{-}$ cells (R2) were gated as before and these cells were analysed for expression of surface molecules and IFN-γ. Representative dotplots are shown for each group.

The $CD8^{hi}TCR1^{-}IFN\text{-}\gamma^{+}$ T cells in the LN predominantly expressed CD45RO, CD45RA, CD62L, CD3, CD27, CD28 and lacked CD25. A small population of $CD8^{hi}TCR1^{-}IFN\text{-}\gamma^{+}$ T cells in the LN expressed CD25 and lacked CD27, CD28, CD45RA and CD62L (Fig 4.4.7B). In the BAL the $CD8^{hi}TCR1^{-}IFN\text{-}\gamma^{+}$ T cells predominantly express CD3, CD45RO, CD27 and CD28 but lack expression of CD45RA and CD62L. Compared to the LN a greater proportion of $CD8^{hi}TCR1^{-}IFN\text{-}\gamma^{+}$ T cells in the BAL express the activation marker CD25. In both the LN and BAL a small percentage of $CD8^{hi}TCR1^{-}IFN\text{-}\gamma^{+}$ T cells are $CD45RA^{+}$, $CD27^{-}$ and $CD28^{-}$ (Fig 4.4.7B).

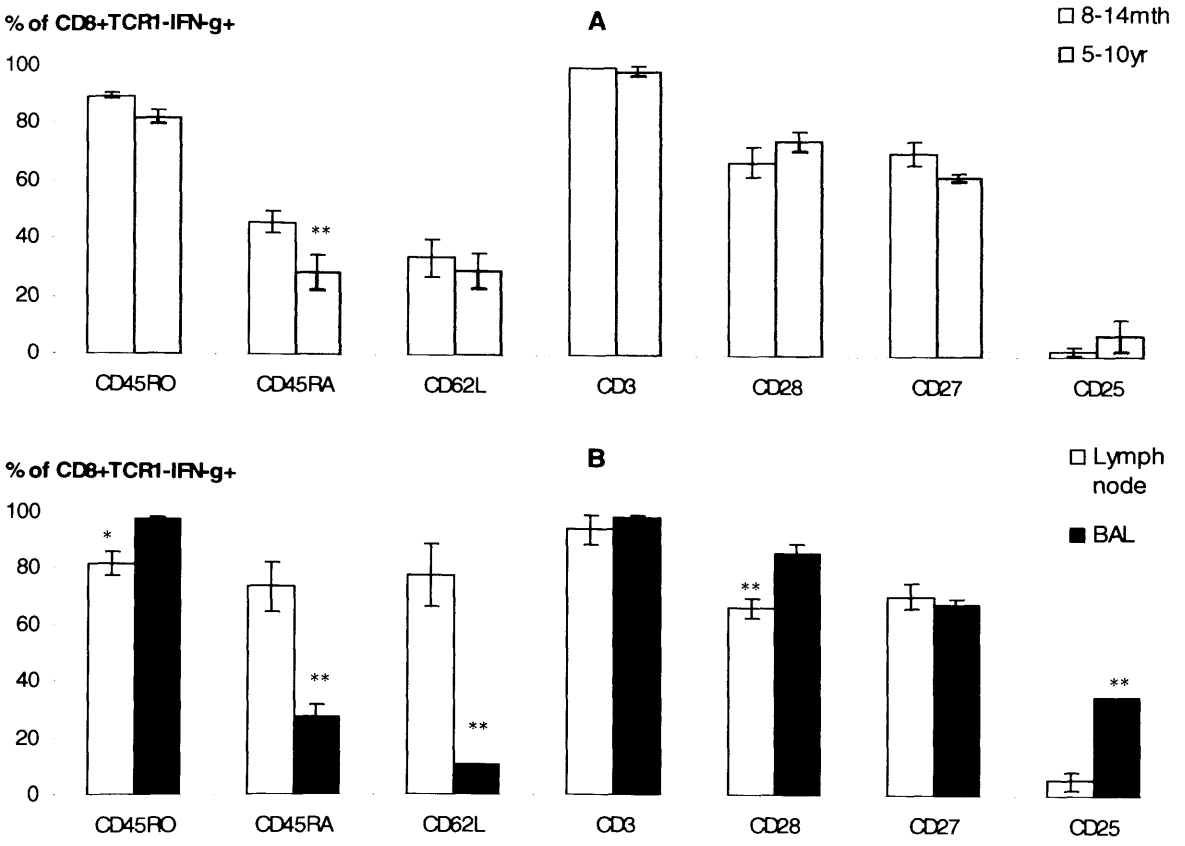


Figure 4.4.7 Surface phenotype of IFN-γ expressing $CD8^{hi}TCR1^{-}$ T cells present in blood (A) and in LN and BAL (B). PBMC (A) and cells isolated from LN and BAL (B) were stimulated for 5hrs with PMA/I/BFA or BFA alone. Cells were then stained with antibodies to CD8, TCR1 and one of the following CD62L, CD45RA, CD45RO, CD28, CD27 and CD25 before being fixed, permeabilised and stained with an antibody which recognises bovine IFN-γ. Gates were placed around the $CD8^{hi}TCR1^{-}$ IFN-γ^{+}T cells and the expression of surface molecules on these cells was investigated. The mean and standard deviation are shown for at least three animals per group. Differences between adjacent age groups or between the LN and BAL that are statistically significant is shown on the older of the two groups or on the BAL as * $p<0.01$, ** $p<0.001$.

4.5 Discussion

In order to investigate the development of memory $CD8^+$ T cell responses in cattle, initial experiments focused on identifying the pattern of surface molecules expressed by naïve, effector and memory $CD8^+$ T cells. At the start of this work information regarding expression of cell surface molecules on $CD8^+$ T cells in cattle was limited. Therefore, the panel of cell surface molecules used in human and murine studies to define different subsets of $CD8^+$ T cells, where reagents were available in cattle, were used in this study.

The results show that the cell-types present in cattle that express the CD8 co-receptor these include γδ T cells ($CD3^+TCR1^+$), NK cells ($CD3^-TCR1^-$) and a population of $CD3^+TCR1^-$ T cells most likely to be the αβ $CD8^+$ T cells. Flow cytometic analysis demonstrated that the $CD3^-$ cells expressed low levels of the CD8 co-receptor. It has been previously reported that the percentage of $WC1^+$ γδ T cells present in blood decreases with age, whereas the results of this study show that the percentage of $CD8^+$ γδ T cells increased with age. It is possible that these two subsets of cells represent γδ T cells in different differentiation states with different functions. Further investigation of expression of cell surface and effector molecules by the different subsets of γδ T cells will be required to confirm this. However, in this study only $CD8^{hi}TCR1^-$ T cells were analysed for expression of cell surface and effector molecules.

It was found that the proportions of $CD8^{hi}TCR1^-$T cell subsets present in blood changes with age of the animal. The percentages of $CD8^{hi}TCR1^-$T cells which express CD45RA, CD62L, CD27 and CD28 decreased with age whereas those expressing CD45RO and CD25 gradually increased with age. These findings agree with data on age-associated changes in the composition of the $CD8^+$ T cell population in human studies (Aldhous, Raab et al. 1994; Brzezinska, Magalska et al. 2004).

The migratory capacity of a naive T cell is limited compared to that of effector or memory T cells as the later have acquired the ability to migrate into non-lymphoid tissue such as the lung and liver (Masopust, Vezys et al. 2001). Naive T cells and a subset of memory T cells express CD62L which mediates specific adhesion to peripheral LN vascular addressins, which target T cells to areas of high antigen concentration within LN and therefore these T cells circulate mainly from the blood to the lymphoid tissue (Sallusto, Lenig et al. 1999; Sallusto, Geginat et al. 2004). In

contrast, expression of CD62L is down-regulated by effector and effector memory T cells as these cells migrate mainly to inflamed peripheral tissues.

In human and mouse studies, different subsets of $CD8^+$T cells have been shown to exhibit different patterns of recirculation, on this basis the populations of bovine $CD8^+$ T cells present in the lung and LN was investigated. It was demonstrated that $CD8^{hi}TCR1^-$T cells present in the BAL express a memory/effector phenotype being $CD45RO^+$, $CD25^+$, $CD62L^-$ and $CD45RA^-$. The presence of $CD8^{hi}TCR1^-$T cells displaying a primed phenotype in the BAL is not unexpected as only T cells that have been primed express the necessary molecules to be able to extravasate into the lung.

In contrast to the BAL, the $CD8^{hi}TCR1^-$T cells the LN expressed cell surface molecules associated with a naïve phenotype being mainly $CD45RA^+$. $CD62L^+$, $CD25^-$, $CD27^+$and $CD28^+$ with the exception of a small population of cells that expressed CD45RO.

Further investigation of the age-associated changes in the proportions of $CD8^{hi}TCR1^-$T cell subsets in blood, showed an increase in the percentage of: $CD45RO^+CD62L^-$, $CD45RO^+CD45RA^-$, $CD45RO^+CD45RA^+$, $CD45RO^+CD27^+$, $CD45RO^+CD28^{+/-}$, $CD45RO^+CD3^+$, $CD45RO^+CD25^{+/-}$. The observed increases further substantiate the idea that the proportion of effector and effector memory $CD8^{hi}TCR1^-$T cells in blood increase with age. Interestingly, a population of $CD8^{hi}TCR1^-CD45RO^-CD27^-$ T cells were observed in older animals and were not evident in the two younger age groups. Loss of expression of CD27 occurs after repeated antigen stimulation (Kuijpers, Vossen et al. 2003; Baars, Sierro et al. 2005). The lack of expression of both CD45RO and CD27 and the appearance of this population in old animals suggests that this may be a population of CD45RA primed cells in cattle.

The largest decreases with age were in the $CD8^{hi}TCR1^-$ T cells that express a naïve phenotype: $CD45RO^-CD45RA^+$, $CD45RO^-CD62L^+$, $CD45RO^-CD27^+$ and $CD45RO^-CD28^+$. In agreement with Hong et al 2004 the proportion of central memory cells, defined in cattle as being $CD8^{hi}TCR1^-CD45RO^+CD62L^+$, did not change significantly with age.

The results demonstrate that the predominant population of $CD8^{hi}TCR1^-$ T cells in the BAL resemble the effector memory T cell population described in human and mice, as they were found to be $CD45RO^+CD62L^-$, $CD45RO^+CD45RA^-$, $CD45RO^+CD28^+$, $CD45RO^+CD27^{+/-}$ and $CD45RO^+CD25^{+/-}$. The large proportion of $CD8^{hi}TCR1^-$

$CD45RO^+CD25^+$ T cells in the BAL suggests that these cells are responsive to IL-2 and could potentially be activated in a bystander manner independent of antigen. Interestingly, there is a high percentage of $CD8^{hi}TCR1^-$T cells in the BAL which express CD45RO but lack expression of CD27 implying that these cells have been repeatedly stimulated but are not terminally differentiated as the majority of the $CD8^{hi}TCR1^-$T cells in the BAL are $CD45RO^+CD28^+$.

Smaller populations of $CD8^{hi}TCR1^-$ T cells were present in the BAL, include $CD8^{hi}TCR1^-CD45RO^-CD45RA^+$ T cells which may represent recently activated T cells or a population of primed T cells that have lost expression of CD45R0 and re-expressed CD45RA. There was also a small population of $CD8^{hi}TCR1^-CD45RO^+CD45RA^+$ T cell it is possible that these represent effector T cells that are in the process of becoming memory T cells.

A population of $CD8^{hi}TCR1^-CD45RO^+CD62L^+$ T cells were present in the BAL, this subset resembles the central memory T cells described in humans and in mice (Sallusto, Lenig et al. 1999; Sallusto, Geginat et al. 2004). Central memory cells home preferentially to the LNs, thus further analysis of the $CD8^{hi}TCR1^-CD45RO^+CD62L^+$ T cell in the BAL is required. Although many models have been proposed the pathway of differentiation of effector cells into memory cells remains unclear. It is possible that these central memory cells in the BAL may have been generated from effector cells and are on their way to the LNs. The presence of a population of central memory cells in the periphery maybe advantageous as there would be no need to wait for the antigen presenting cell to migrate into the lymphoid nodes. These cells could be activated and give rise to effector cells within the tissue without requiring cell migration from the LN to the site of infection or inflammation.

The majority of the $CD8^{hi}TCR1^-$T cells in the LN were found to be $CD45RO^-$ $CD45RA^+$, $CD45RO^-CD62L^+$, $CD45RO^-CD28^+$, $CD45RO^-CD25^-$ and $CD45RO^-$ $CD27^+$. This pattern of expression suggests that the $CD8^{hi}TCR1^-$T cells in the LN are mostly naive T cells. Similar to the findings in the BAL there are smaller populations of cells in the LN which may represent T cells at various stages of differentiation. There were small subsets of $CD8^{hi}TCR1^-$T cells that were: $CD45RO^+CD3^+$, $CD45RO^+CD62L^+$, $CD45RO^+CD45RA^{-/+}$, $CD45RO^+CD27^{+/-}$, $CD45RO^+CD28^{+/-}$. All $CD8^{hi}TCR1^-CD45RO^+$ T cells in the LN were found to express CD62L. Studies in humans and mice have demonstrated that central memory cells reside in the LN and are $CD45RO^+CD62L^+CCR7^+$ (Sallusto, Lenig et al. 1999; Sallusto, Geginat et al. 2004). It

is likely that a population of central memory cells is present bovine LN and can be identified as being $CD8^{hi}TCR1^{-}CD45RO^{+}CD62L^{+}$.

The release of cytoplasmic granules containing perforin, granzymes and granulysin is one mechanism employed by cytotoxic cells to kill target cells. Perforin is thought to polymerise to form a pore in the cell membrane of the target cell and facilitates the release of granzymes and granulysin into the target cell. Granzymes are serine proteases, which once inside the cell activate capases and subsequent fragmentation of DNA, with the end result being death of the target cell. The ability of a $CD8^{+}$ T cells to express perforin is acquired after priming and differentiation of a naïve $CD8^{+}$ T cell into an effector and memory T cells (Bachmann, Barner et al. 1999; Veiga-Fernandes, Walter et al. 2000).

It is known that T cells at different stages of differentiation express disparate levels of perforin (Rufer, Zippelius et al. 2003). The sole use of expression of surface molecules to define subsets of $CD8^{+}$ T cells is limited. Therefore expression of effector molecules, perforin and IFN-γ was also investigated. It was observed that the percentage of $CD8^{+}TCR1^{-}$ T cells in blood that expressed perforin increased with age. Perforin was found to be expressed by cells with an effector or effector memory phenotype. This is in agreement with the early observations that the proportion of $CD8^{hi}TCR1^{-}$T cells expressing an effector or effector memory phenotype in blood increases with age. It is important to state that expression of perforin by bovine $CD8^{hi}TCR1^{-}$T cells was analysed ex vivo without any prior stimulation, implying that perforin is constitutively expressed by some $CD8^{hi}TCR1^{-}$T cells in cattle. Further investigation is required to determine if expression of perforin ex vivo identifies cells that are able to kill.

A higher percentage of $CD8^{hi}TCR1^{-}$ T cells expressed perforin in the BAL, than in the LN. This coincides with the finding that the $CD8^{hi}TCR1^{-}$ T cells in BAL express effector or effector memory surface phenotype whereas in the LN these cells mostly expressed a surface phenotype similar to naïve T cells.

In the blood, perforin was found to be expressed by $CD8^{hi}TCR1^{-}$T cells that were: $CD62L^{+/-}$, $CD45RA^{+/-}$, $CD28^{+/-}$, mostly $CD45RO^{+}$, $CD27^{+}$ and $CD25^{-}$. In these subsets the percentage of perforin expressing $CD8^{hi}TCR1^{-}$T cell increased with age.

An increase in perforin expressing $CD8^{hi}TCR1^{-}CD45RA^{+}$, $CD8^{hi}TCR1^{-}CD45RO^{+}$and to a lesser extent $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells suggests the presence of effector cells in blood that are either $CD45RA^{+}CD45RO^{-}$ and $CD45RA^{+}CD45RO^{+}$. Furthermore, the increases in perforin-expressing $CD8^{hi}TCR1^{-}CD28^{-}$ and to lesser extent $CD8^{hi}TCR1^{-}CD27^{-}$ suggests that the proportion of highly differentiated $CD8^{+}$ T cells that express perforin accumulates during the lifetime of an animal.

Interestingly, an increase in the percentage of $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells that lack perforin was observed. The effector functions of these cells are unclear but it is possible that they have down-regulated perforin expression, or that they not received adequate stimulation to acquire effector functions.

Central memory cells ($CD45RO^{+}CCR7^{+}CD62L^{+}$) have been shown to exhibit a degree of effector functions in human and mice (Wherry, Teichgraber et al. 2003). The finding that some $CD8^{hi}TCR1^{-}CD62L^{+}$ T cells present in blood express perforin suggests that these cells may represent a population of central memory cells in cattle.

In the BAL the perforin expressing cells were found predominantly in the following $CD8^{hi}TCR1^{-}$T cell subsets: $CD62L^{-}$, $CD45RA^{-}$, $CD45RO^{+}$, $CD28^{+}$, $CD27^{+}$ and $CD25^{+}$. Minor populations of $CD8^{hi}TCR1^{-}perforin^{+}$ T cells were also evident in BAL which included cells that expressed CD45RA. These may represent a population of RA primed $CD8^{+}$T cells described in human studies. A minor population of $CD8^{hi}TCR1^{-}Perforin^{+}$ T cells that has lost both CD27 and CD28 was present in the BAL, similar to blood, suggesting that these cells are highly differentiated. The presences of these cells in the respiratory tract may reflect the continuous exposure to antigens. In addition, the cells present in the BAL may also have been repeatedly stimulated in a bystander manner because of their activated status.

The LN was found to be comprised mostly of naïve T cells but also contains some memory cells and perhaps a small population of recently activated T cells. The small population of $CD8^{hi}TCR1^{-}Perforin^{+}$ T cells in the LN were; $CD62L^{+}$, $CD45RO^{+}$, $CD27^{+}$, $CD28^{-}$ and $CD45RA^{+/-}$. These findings are puzzling as the expression of CD45RO and CD62L on the $CD8^{hi}TCR1^{-}Perforin^{+}$ T cells is suggestive of central memory cells. One the other hand approximately half of these $perforin^{+}$ cells express CD45RA, therefore these cells may represent recently activated T cells in transition from effector to memory or a population of CD45RA primed cells.

Interestingly, the $CD8^{hi}TCR1^{-}perforin^{+}$ cells in the LN expressed CD27 but lacked CD28 suggesting that these cells have been primed and are differentiated as they lack

CD28 but have not been repeatedly stimulated by antigen as they have retained expression of CD27. These results support the idea that there are memory cells in bovine LN that upon activation, respond to antigen, proliferate and differentiate into effector cells.

As with expression of perforin, the percentage of $CD8^{hi}TCR1^{-}$T cells in blood that express IFN-γ after mitogen stimulation increases with age. Due to the lack of young animals the data does not contain a neonate age group. An age-related increase in the percentage of $CD8^{+}$ T cells expressing IFN-γ has been reported in humans and mice (Chipeta, Komada et al. 1998; Nociari, Telford et al. 1999). In agreement with the perforin data, the percentage of $CD8^{hi}TCR1^{-}IFN\text{-}\gamma^{+}$ T cells after mitogen stimulation was higher in the BAL compared to the LN.

In blood, IFN-γ was found to be expressed by the $CD8^{hi}TCR1^{-}$T cells that were: $CD62L^{+/-}$, $CD45RA^{+/-}$, $CD28^{+/-}$, $CD27^{+/-}$, $CD45RO^{+}$ and $CD25^{-}$. The percentage of IFN-γ expressing $CD8^{hi}TCR1^{-}$ T cells that increased with age were found to be: $CD62L^{-}$, $CD45RA^{-}$, $CD45RO^{+}$, $CD28^{+}$, $CD27^{+/-}$and $CD25^{+/-}$. In humans, it has been previously shown that the proportion of $CD8^{+}CD28^{+}$ T cells in blood that express IFN-γ after mitogen stimulation was increased in patients with chronic hepatitis C infection (Murata, Nabeshima et al. 2002). The same study reported a related increase in the percentage of $CD8^{+}$T cells expressing a memory phenotype ($CD45RA^{-}CD28^{+}$).

A small population of $CD8^{hi}TCR1^{-}CD45RO^{-}IFN\text{-}\gamma^{+}$ T cells were evident in blood from the older animals, these cells may represent a bovine equivalent of CD45RA primed T cells.

In the BAL, IFN-γ was expressed by $CD8^{hi}TCR1^{-}$T cells that resembled a population of effector or effector memory cells as they were: $CD62L^{-}$, $CD45RA^{-}$, $CD45RO^{+}$, $CD28^{+}$, $CD27^{+/-}$ and $CD25^{+/-}$. The minor population of $CD8^{+}TCR1^{-}CD45RA^{+}$ $IFN\text{-}\gamma^{+}$ T cells found in the BAL suggest that these cells are either a population of RA-primed cells or recently activated T cells.

The IFN-γ expressing $CD8^{hi}TCR1^{-}$T cells in the LN were similar to either central memory T cells or recently activated T cells as they were mostly: $CD62L^{+}$, $CD45RA^{+}$, $CD45RO^{+}$, $CD28^{+}$, $CD27^{+}$ and $CD25^{-}$.

It appears that a population of RA-primed $CD8^+$ T cells are present in aged cattle that are homologous to the RA-primed cells that arise in aged individuals and in many chronic infections, as a small proportion of bovine $CD8^{hi}TCR1^-CD45RA^+$ T cells were found to express perforin and IFN-γ. In cattle, the frequency of RA-primed cells increased with age in the blood and were found at a similar percentage in the LN and BAL. The function and antigen specificity of bovine $CD8^{hi}TCR1^-CD45RA^+$ primed T cells has yet to be investigated but in human studies this population arises mainly after chronic antigen exposure. Previous work in cattle demonstrated that during infection with *Theileria parva* the responding $CD8^+$ cells could be found among the $CD45RO^+$ and $CD45RO^-$ subsets (Bembridge, MacHugh et al. 1995). Although this study suggests that CD45RA primed cells may arise during *Theileria parva* infection, these results must be interpretated with caution because the total $CD8^+$ cell population was used and our data show that it contains a large proportion of γδ T cells and NK cells.

The predominant population of $CD8^{hi}TCR1^-$ T cells that expresses IFN-γ and perforin in the BAL express a different pattern of surface molecules compared to the LN.

These cells in the BAL resemble effector or effector memory cells whereas in the LN they resemble central memory T cells. In blood, the percentage of $CD8^{hi}TCR1^-$ T cells expressing perforin and IFN-γ increased with age, this coincides with the observed increase in the percentage of $CD8^{hi}TCR1^-$ T cells expressing an effector or effector memory phenotype.

The results of this study provide evidence for the presence of at least two subsets of memory $CD8^+$T cells in cattle similar to those described in human and murine studies defined by location and expression of CD45RO and CD62L as being central ($CD45RO^+CD62L^+$) and effector ($CD45RO^+CD62L^-$) memory cells. These two memory subsets in cattle were also found to be heterogeneous in their expression of CD27 and CD28, again this is in concordance with reports in humans and mice.

In summary these observations show that the frequency of $CD8^{hi}TCR1^-$T cells that express an effector or memory surface phenotype and effector mediators such as perforin and IFN-γ increase in blood with age and are present at a higher frequency in the BAL compared to LN.

CHAPTER FIVE: RESULTS

Development of $CD8^+$ T cell responses after BCG vaccination of cattle

5.1 Introduction

Mycobacterium bovis bacilli Calmette-Guerin (BCG) is the only licensed vaccine against human TB. Since its introduction in 1921, BCG has been administered to more than 3 billion people. Vaccination with BCG is known to be protective against childhood forms of tuberculosis (Rodrigues, Diwan et al. 1993) but its efficacy against adult pulmonary tuberculosis has been shown to be variable (Colditz, Brewer et al. 1994; Fine 1995; Brewer 2000).

To date there are no approved TB vaccines for use in cattle. BCG vaccination of cattle is known to provide a degree of protection against tuberculosis caused by infection with *M. bovis* (Buddle, de Lisle et al. 1995; Buddle, Keen et al. 1995; Hope, Thom et al. 2005). Vaccinated cattle have reduced lesion scores, pathology and mycobacterial load. The variability in the efficacy of BCG in protecting cattle may be attributed to the use of different sub-strains, doses and routes of administration in the different protection trials. Another variable that has to be taken into account is animal to animal variation as the capacity of animals to respond to vaccination is influenced by many factors which include genetic background, immunocompetence, exposure and reactivity to environmental mycobacteria.

The presence of mycobacteria reactive $CD8^+$ T cells has been demonstrated in BCG vaccinated individuals in human studies. The responding $CD8^+$ T cells up-regulated expression of CD25, IFN-γ, TNF-α and perforin after *in vitro* culture with BCG. These BCG-reactive $CD8^+$ T cells were also shown to specifically lyse BCG-infected cells (Turner and Dockrell 1996; Smith, Malin et al. 1999). Addition of inhibitors of the Golgi-ER processing pathway, phagocytosis and the proteosome activity to the cultures all reduced the level of killing to background levels (Smith, Malin et al. 1999).

BCG vaccination of cattle has been shown by many groups to induce the production of IFN-γ secreting mycobacteria-specific T cells (Buddle, Keen et al. 1995; Hope, Kwong et al. 2000; Buddle, Wedlock et al. 2003; Hope, Thom et al. 2005). Monitoring of the immune response induced by BCG vaccination of cattle, most commonly involves the culture of either whole blood or PBMC with PPD-B and has been shown to peak at 2-4 wks post-vaccination. The use of a protein antigen suggests that the mycobacteria specific-T cells detected in these studies are most likely to be $CD4^+$ T cells.

Hope et al showed that mycobacteria-reactive $CD4^+$ T cells and $CD8^+$ cells from BCG-vaccinated animals (ages 6mths-4yrs) proliferated after cultured with BCG-infected monocyte-derived DCs. Furthermore both $CD8^+$ cells and $CD4^+$ T cells from non-vaccinated animals were also found to proliferate under these conditions although to a lesser extent (Hope, Kwong et al. 2000). It has also been reported that a population of $CD8^+$ cells from non-vaccinated gnotobiotic calves proliferate and produce IFN-γ after culture with BCG-infected DC. The population of cells used in this study were total $CD8^+$ cells, which was shown in chapter 4 to be comprised of γδ T cells ($CD3^+TCR1^+$), NK cells ($CD3^-TCR1^-$) and αβ T cells ($CD3^+TCR1^-$). Flow cytomteric analysis demonstrated that the majority of responding $CD8^+$ cells were $CD3^-$ and $TCR1^-$ and the small population of responding $CD8^+CD3^+$ cells expressed the γδ TCR. It was also shown that the response to the BCG-infected DC was not MHC restricted therefore it is unlikely that the study by Hope et al were measuring a $CD8^+$αβ T cell responses (Hope, Sopp et al. 2002).

Due to the questionable efficacy of BCG vaccination in cattle and in humans, future vaccination strategies are being aimed at providing a heterologous prime-boost strategy. The continued use of BCG in humans is favourable as it protects against childhood forms of tuberculosis and is safe, stable and cheap to produce. Due to these qualities it is seen as the benchmark to which all other TB vaccines must exceed in terms of level of protection. Current thinking is to keep the pre-existing strategy of vaccination at birth with BCG and then boost with another vaccine at during early adulthood. It has been shown that vaccination with BCG and then boosting with BCG did not provide a greater level of protection compared to just giving a single BCG vaccine. It is thought that the BCG boost is cleared by the memory response induced by the initial BCG vaccination before it can

stimulate a substantial immune response. Furthermore in cattle revaccination with BCG was found be contraindicative when the animals were infected with *M. bovis* (Buddle, Wedlock et al. 2003). Other antigens that have been used to boost BCG vaccine have been discussed in the introduction. The most promising prime boost strategy against TB in humans involves priming with BCG and boosting with MVA-Ag85, however this protocol induced mostly $CD4^+$ T cells (McShane, Pathan et al. 2004).

Parallel studies performed in cattle have shown that vaccination with BCG and boosting with MVA-Ag85 induces a stronger immune response against a wider repertoire of Ag85 16 mer peptides compared to priming with BCG and boosting with BCG. $CD4^+$ and $CD8^+$ T cells from animals given BCG and boosted with MVA-Ag85A expressed IFN-γ after overnight culture with PPD-B (Vordermeier, Rhodes et al. 2004).

More recently it has been reported that intranasal vaccination of mice with recombinant adenovirus expressing Ag85A (Ad-Ag85A) protected mice against tuberculosis (Santosuosso, Zhang et al. 2005). Similar experiments were repeated in cattle in which animals were given BCG and boosted with Ad-Ag85. It was found that this heterologous prime-boost strategy induced a strong cellular immune response (Vordermeier, Huygen et al. 2006).

In order to design vaccines to enhance the protective efficacy of BCG, a clearer understanding of the immune response induced by vaccination with BCG is needed. In order for the booster vaccine to be effective it should target specific arms of the immune response against immunodominant antigens. A clearer understanding of the immune response induced by BCG may also provide clues as to why BCG vaccination confers protection against disease in some animals but not in others.

5.2 Analysis of the immune response induced by BCG vaccination

To determine whether mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells can be detected after vaccination with BCG, blood samples were taken from two 3 mth-old animals before and after vaccination with BCG. $CD8^+$ cells were isolated from PBMC using the MACS sorting system and stained with an antibody to the γδ T cell receptor (TCR1). The cells were then sorted on a Moflo cell sorter into $CD8^{hi}TCR1^-$ and $CD8^{hi}TCR1^+$. Expression of CD8 identifies two populations of cells into $CD8^{hi}$ and $CD8^{lo}$. The population $CD8^{lo}$ was

found to contain a large proportion of cells which lack expression of CD3, whereas the $CD8^{hi}$ population were found to be largely $CD3^+$ (98%). The $CD8^{hi}TCR1^-$, $CD8^{hi}TCR1^+$, total $CD8^+$ cells and PBMC were stimulated for 5 days *in vitro* with uninfected, PPD-B-pulsed or BCG infected Mϕ. Mycobacteria-reactive T cells were detected by proliferation measured using ^3H TdR incorporation.

Mycobacteria-reactive cells in the PBMC could be detected at 2 wks post-vaccination in the both animals (Fig. 5.2.1 and 5.2.2). At 4 wks post-vaccination, the total $CD8^+$ cells from animal 195 proliferated in response to PPD-B pulsed Mϕ, suggesting the development of mycobacteria-reactive $CD8^+$ cells (Fig. 5.2.1). Prior to vaccination, the total $CD8^+$ cells from animal 198 proliferated in response to BCG and PPD-B. Vaccination with BCG reduced this response indicating that perhaps the detected response was due to a population of innate immune cells such as NK cells or γδ T cells (Fig 5.2.2).

In previous experiments, stimulation of the total $CD8^+$ cell population with PPD-B resulted in a large expansion of $CD8^+$γδ T cells. These $CD8^+$γδ T cells significantly out grew the $CD8^+TCR1^-$ T cells. Therefore the $CD8^{hi}$ γδ T cells were included in this experiment to investigate if these cells when sorted respond to either PPD-B pulsed or BCG-infected Mϕ. It is shown in figures 5.2.1 and 5.2.2 that no response to mycobacteria was detected in these $CD8^{hi}$ γδ T cells. It is possible that $CD8^{hi}$ γδ T cells only respond to antigen when cultured together with other subsets of responding T cells and may be reacting to a cytokine produced. It is also possible that the mycobacteria-reactive $CD8^+$γδ T cells express low levels of CD8 and were therefore excluded from these experiments.

At 4 wks post-BCG vaccination mycobacteria-reactive $CD8^{hi}TCR1^-$ (αβ) T cells were detected in blood from both animals. These cells proliferated after culture with BCG-infected Mϕ but not PPD-B-pulsed Mϕ. In the time-frame of this experiment the proliferative response of the $CD8^{hi}TCR1^-$ (αβ) T cells from both animals peaked at 8 wks post-vaccination (Fig 5.2.1 and 5.2.2). A further time-point was analysed in animal 195

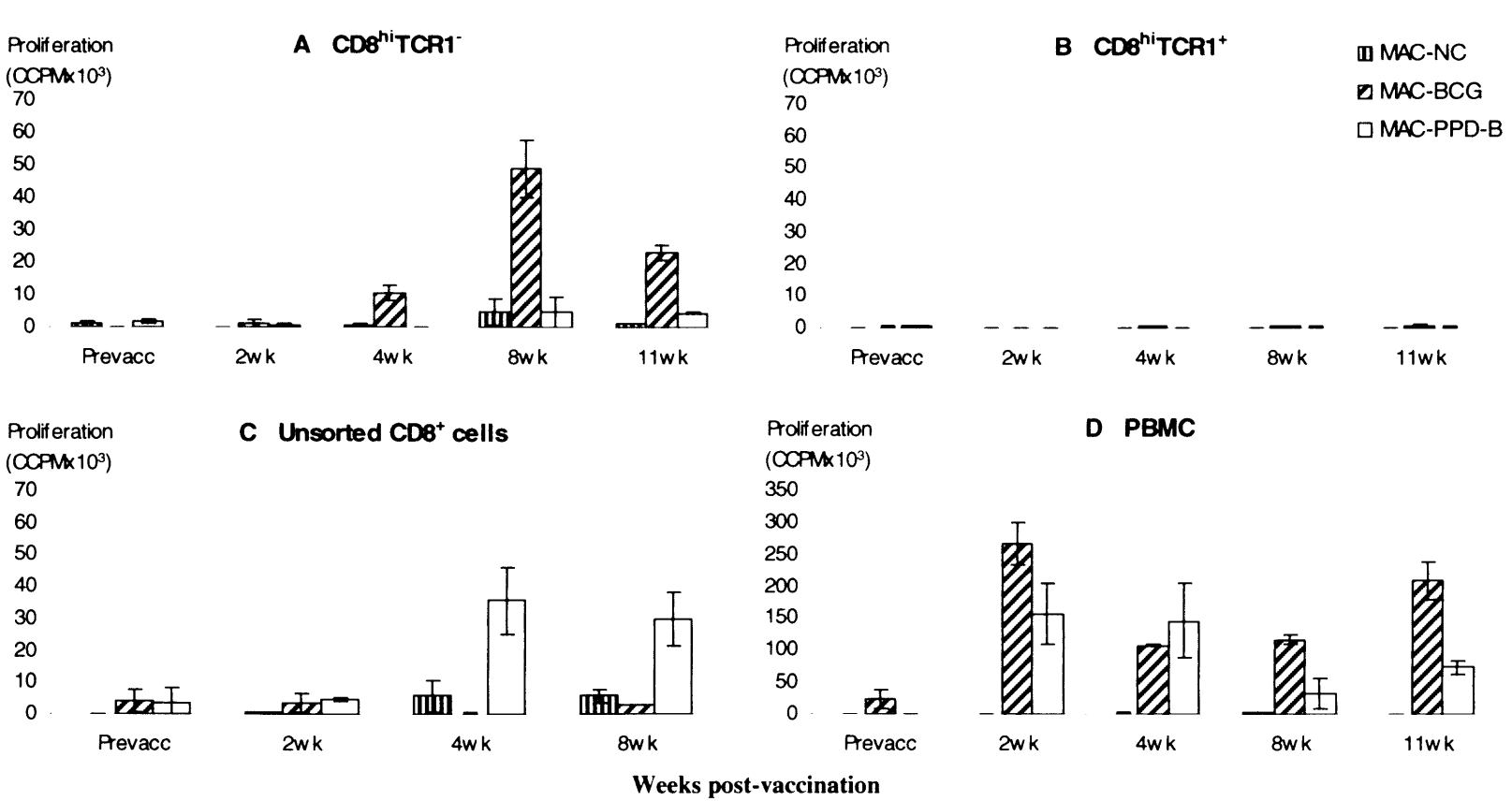


Figure 5.2.1 Vaccination with BCG induces $CD8^+$ T cells and PBMC to proliferate in response to BCG infected Mϕ. CD8 expressing cells were isolated from PBMC using a paramagnetic sorting system. $CD8^+$ T cells were then stained with antibody to the γδ TCR (TCR1) and sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-$ and $CD8^{hi}TCR1^+$. The two MoFlo-sorted subsets $CD8^{hi}TCR1^-$ (A) and $CD8^{hi}TCR1^+$ (B), unsorted $CD8^+$cells (C) and PBMC (D) were cultured for 5 days *in vitro* with uninfected, uninfected with PPD-B (10μg/ml) or BCG-infected Mϕ (MAC). To measure proliferation, cells were labelled with tritiated thymidine (^3H) for 8-16 hr before being harvested and the amount of thymidine incorporated into the cells is shown as CCPM. The mean and standard deviation is shown fro triplicate samples from one BCG-vaccinated animal (195).

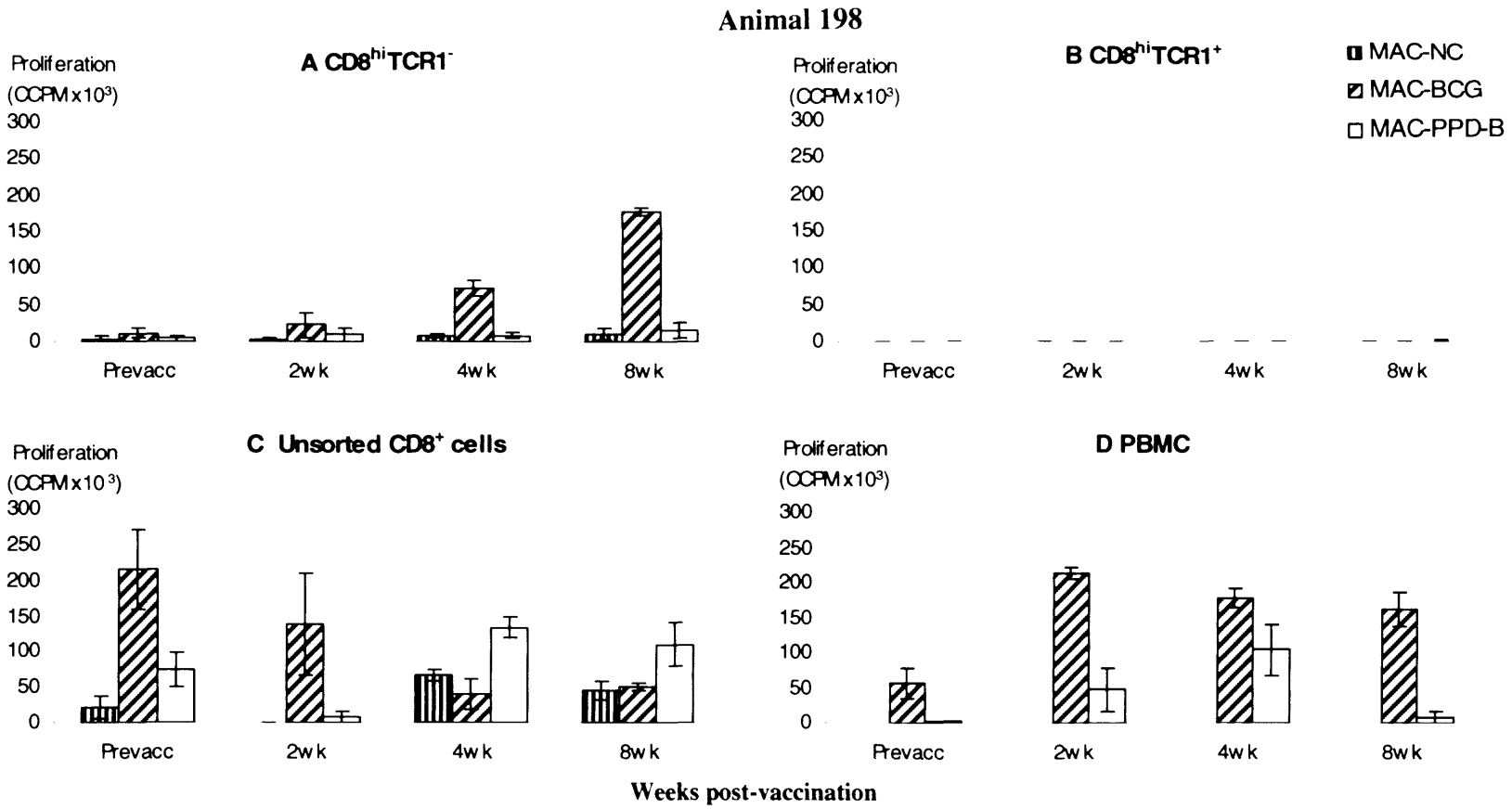


Figure 5.2.2 Vaccination with BCG induces $CD8^+$ T cells and PBMC to proliferate in response to BCG-infected Mϕ. CD8 expressing cells were isolated from PBMC using a paramagnetic sorting system. $CD8^+$ T cells were then stained with antibody to the γδ TCR (TCR1) and sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-$ and $CD8^{hi}TCR1^+$. The two MoFlo-sorted subsets $CD8^{hi}TCR1^-$ (A) and $CD8^{hi}TCR1^+$ (B), unsorted $CD8^+$cells (C) and PBMC (D) were cultured for 5 days *in vitro* with uninfected, uninfected with PPD-B (10μg/ml) or BCG-infected Mϕ (MAC). To measure proliferation, cells were labelled with tritiated thymidine (^3H) for 8-16 hr before being harvested and the amount of thymidine incorporated into the cells is shown as CCPM. The mean and standard deviation is shown for triplicate samples from one BCG-vaccinated animal (198).

and it showed that the level of proliferation had decreased from 8 wks to 11 wks post-vaccination (Fig 5.2.1).

The lack of a proliferative response in the $CD8^{hi}TCR1^-$ T cells after culture with PPD-B pulsed Mϕ suggests that in this assay the protein antigen PPD-B is not cross-presented on MHC Class I molecules by bovine monocyte-derived Mϕ.

5.3 Analysis of immune responses induced by BCG-vaccination of 6 mth old animals

5.3.1 Characterisation of mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells induced by BCG vaccination

To further characterise the mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells detected in blood after BCG vaccination, blood samples were taken before and at intervals after BCG vaccination from three 6 mth old animals. Analysis was performed on blood from the three animals on the same day at each time-point. The results from chapter 4 suggest that expression of CD45RO on $CD8^+TCR1^-$ T cells identifies a population of effector cells, a large proportion of which express perforin and IFN-γ. Based on these results, $CD8^+$ cells were isolated from PBMC as described in materials and methods and were sorted on a Moflo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. These two sorted $CD8^{hi}$ T cell subsets, PBMC and total $CD8^+$ cells were cultured *in vitro* with uninfected and BCG infected monocyte-derived Mϕ and DC for 5 days. The presence of mycobacteria-reactive T cells was detected by proliferation measured using ^3H TdR incorporation and production of IFN-γ measured by ELISA.

The results show that mycobacteria-reactive T cells can be detected in blood after vaccination with BCG in all three animals. In the three animals, BCG-infected Mϕ were found to be more potent stimulators of proliferation and IFN-γ production in the T cells compared to the BCG-infected DC. No response to mycobacteria was observed in the $CD8^{hi}TCR1^-CD45RO^-$ T cell subset in any of the animals throughout the experiment.

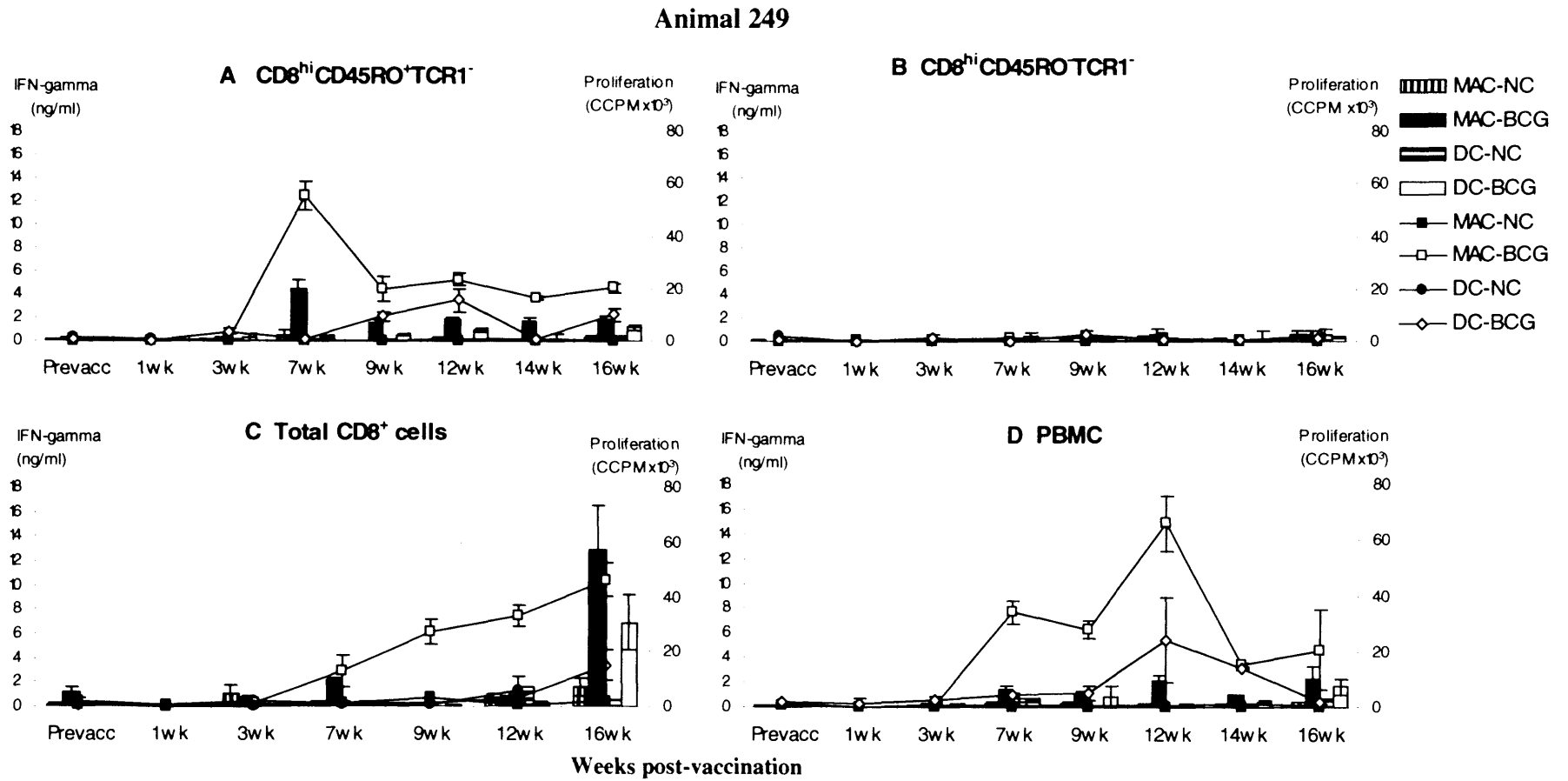


Figure 5.3.1 Vaccination with BCG induces T cells from animal 249 to proliferate and produce IFN-γ after culture with mycobacteria. CD8 expressing cells were isolated from PBMC using MACS isolation beads. $CD8^+$ T cells were then stained with antibody to the γδ TCR (TCR1) and CD45RO before being sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The two MoFlo-sorted subsets $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days *in vitro* with uninfected or BCG-infected Mϕ (MAC) and dendritic cells (DC). Proliferation was measured by uptake of tritiated thymidine (^3H) and is shown as CCPM (line). Production of IFN-γ was measured by ELISA and is shown as ng/ml (column) The mean and standard deviation is shown for triplicate samples from one BCG-vaccinated animal (249).

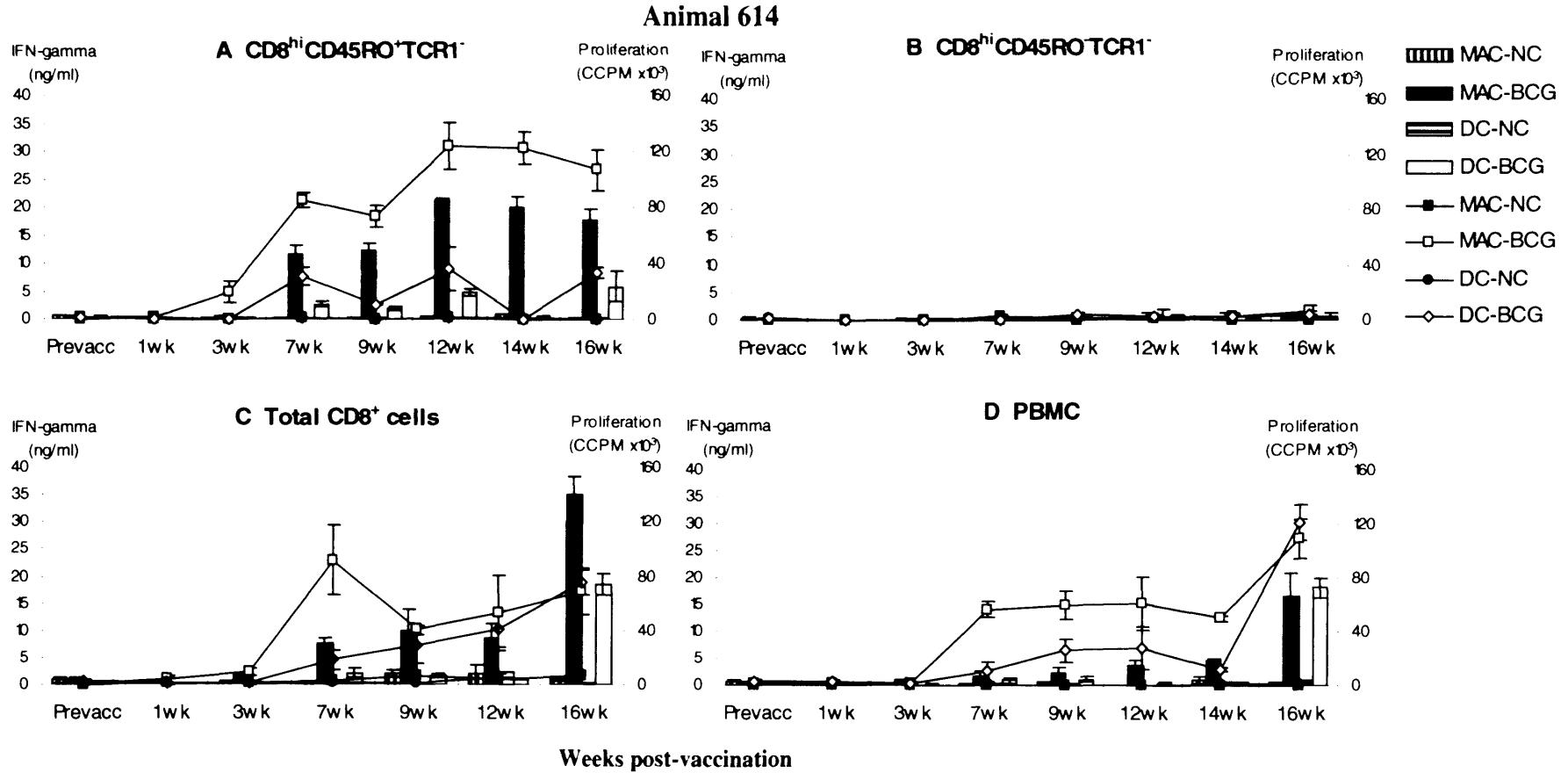


Figure 5.3.2 Vaccination with BCG induces T cells from animal 614 to proliferate and produce IFN-γ after culture with mycobacteria. CD8 expressing cells were isolated from PBMC using MACS isolation beads. $CD8^+$ T cells were then stained with antibody to the γδ TCR (TCR1) and CD45RO before being sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The two MoFlo-sorted subsets $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days *in vitro* with uninfected or BCG-infected Mϕ (MAC) and dendritic cells (DC). Proliferation was measured by uptake of tritiated thymidine (^3H) and is shown as CCPM (line). Production of IFN-γ was measured by ELISA and is shown as ng/ml (column) The mean and standard deviation is shown for triplicate samples from one BCG-vaccinated animal (614).

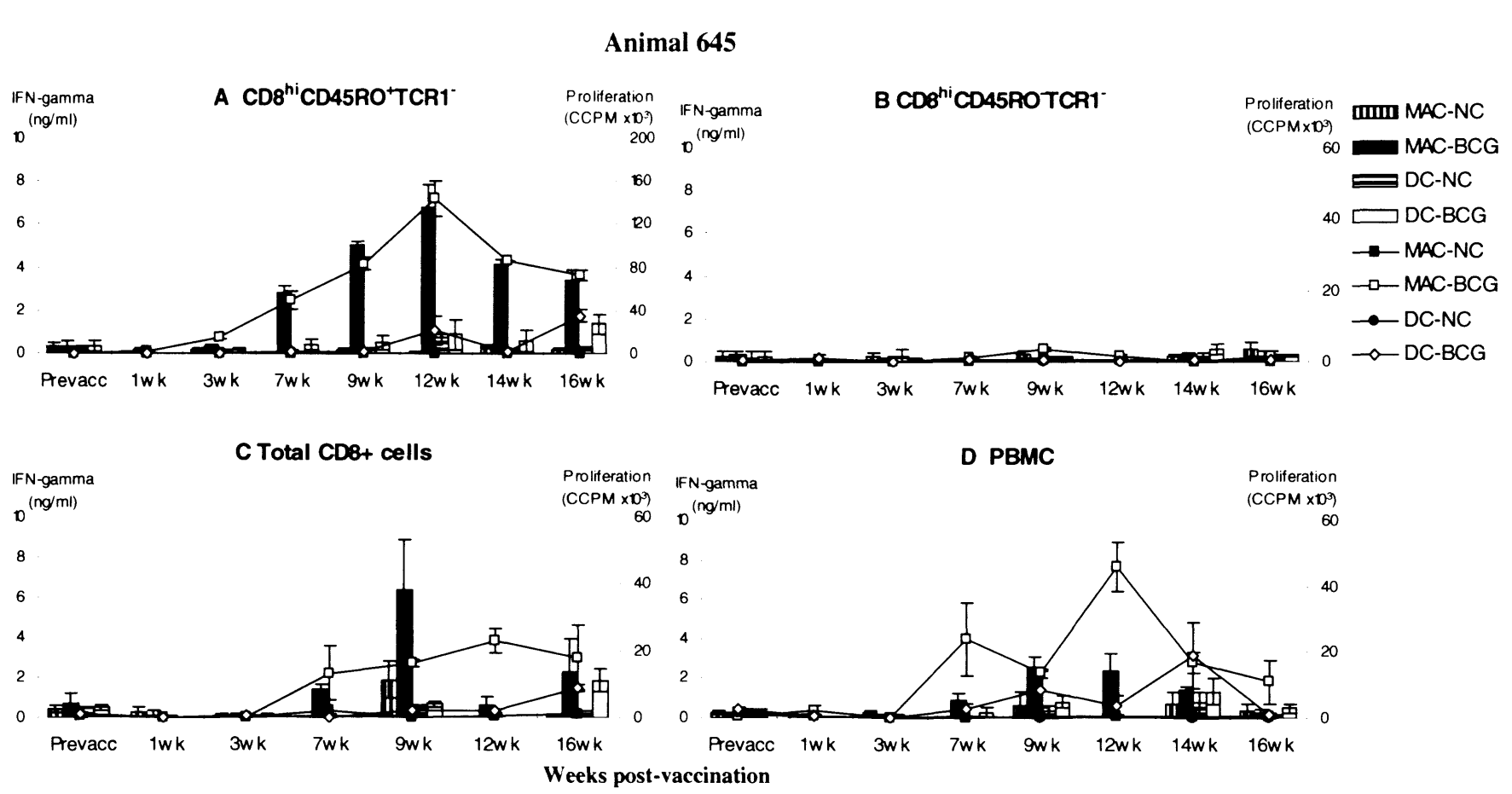


Figure 5.3.3 Vaccination with BCG induces T cells from animal 645 to proliferate and produce IFN-γ after culture mycobacteria. CD8 expressing cells were isolated from PBMC using MACS isolation beads. $CD8^+$ T cells were then stained with antibody to the γδ TCR (TCR1) and CD45RO before being sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The two MoFlo-sorted subsets $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days *in vitro* with uninfected or BCG-infected Mϕ (MAC) and dendritic cells (DC). Proliferation was measured by uptake of tritiated thymidine (^3H) and is shown as CCPM (line). Production of IFN-γ was measured by ELISA and is shown as ng/ml (column) The mean and standard deviation is shown for triplicate samples from one BCG-vaccinated animal (645).

In animal 249, at 7 wks post-vaccination, mycobacteria-reactive $CD8^+TCR1^-CD45RO^+$ T cells proliferated and produced IFN-γ. This response peaked at 7 wks post-vaccination and then declined slightly but was still detectable at 16 wks post-vaccination (Fig 5.3.1A). The mycobacteria-reactive cells were first detected in the total $CD8^+$ cells at 7 wks post-vaccination and the response of these cells gradually increased until 16 wks post-vaccination (Fig 5.3.1C). The observed response in the PBMC after vaccination with BCG peaked at 12 wks post-vaccination and was still detectable at 16wks post-vaccination (Fig. 5.3.1D).

In animal 614, mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells were first detected at 3 wks post-vaccination, and the response of these cells peaked 12wks and was still detectable at 16 wks post-vaccination. The responding $CD8^{hi}TCR1^-CD45RO^+$ T cells produced IFN-γ and proliferated after culture with BCG-infected Mϕ (Fig. 5.3.2A). BCG-reactive cells in the total $CD8^+$ cells were detected at 7 wks post-vaccination by production of IFN-γ and proliferation in response to BCG-infected Mϕ (Fig 5.3.2B). Mycobacteria-reactive cells were detected in PBMC at 7 wks post-vaccination by proliferation to BCG-infected Mϕ or DC and this response was found to peak at 16 wks post-vaccination (Fig.5.3.2D).

Mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells were detected in animal 645 at 7 wks post-vaccination. These cells proliferated and produced IFN-γ after culture with BCG-infected Mϕ. The response of the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells peaked at 12 wks but was still detectable at 16 wks post-vaccination (Fig 5.3.3A). A weaker response to mycobacteria was detected at 7 wks post-vaccination in the total $CD8^+$ cells and PBMC compared to the $CD8^{hi}TCR1^-CD45RO^+$ T cells (Fig 5.3.3A, C and D). The response of the mycobacteria-reactive PBMC peaked at 12 wks and had declined substaintially at 16 wks post-vaccination (Fig 5.3.3D).

Thus, after vaccination with BCG, mycobacteria-reactive cells could be detected in PBMC, total $CD8^+$ cells and $CD8^{hi}TCR1^-CD45RO^+$ T cells in all three animals.

5.3.2 Analysis of mRNA expression of perforin, granulysin and CCR7 by mycobacteria-reactive $CD8^{hi}TCR1^{-}$ T cells

To further investigate effector functions of the mycobacteria-reactive $CD8^{hi}TCR1^{-}$ $CD45RO^{+}$ T cells that develop after vaccination with BCG, RT-PCR was used to analyse expression of perforin, CCR7 and granulysin after stimulation with mycobacteria. Expression of CCR7 and granulysin were analysed by PCR because at present there are no monoclonal antibodies to these molecules in cattle but the sequences are available. Expression of the house-keeping gene β-actin was determined in the samples as a control to show that similar amounts of RNA was present in each sample. The chemokine receptor CCR7 has been used extensively in human studies to define subsets of T cells that differ in effector functions with loss of expression of this molecule being associated with gain of immediate effector functions (Sallusto, Lenig et al. 1999). In addition CCR7 expression has been used to differentiate between central ($CCR7^{+}$) and effector ($CCR7^{-}$) memory $CD8^{+}$ T cells in humans (Sallusto, Geginat et al. 2004). Active peptides of granulysin have been shown to directly lyse mycobacteria and release of this mycobactericidal molecule is one mechanism by which $CD8^{+}$ T cells have been proposed to contribute to the control mycobacterial infections (Stenger, Hanson et al. 1998). Recently a bovine homologue to human granulysin was identified and named Bolysin (Endsley, Furrer et al. 2004). Bolysin mRNA was shown to be expressed by bovine T cells after strong mitogen stimulation. Perforin and granulysin are constituents of cytotoxic granules expressed by cytotoxic T cells. Perforin functions to form pores in the cell membrane of targets and has been shown to be required for the release of granulysin into the target cell (Stenger, Hanson et al. 1998).

The $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cell subsets were sorted from blood from BCG vaccinated animals one the same day at 15 wks post-vaccination and cultured with uninfected or BCG-infected macrophages for 5 days. After which mRNA was extracted from the cells and analysed for expression of β-actin, CCR7, granulysin and perforin.

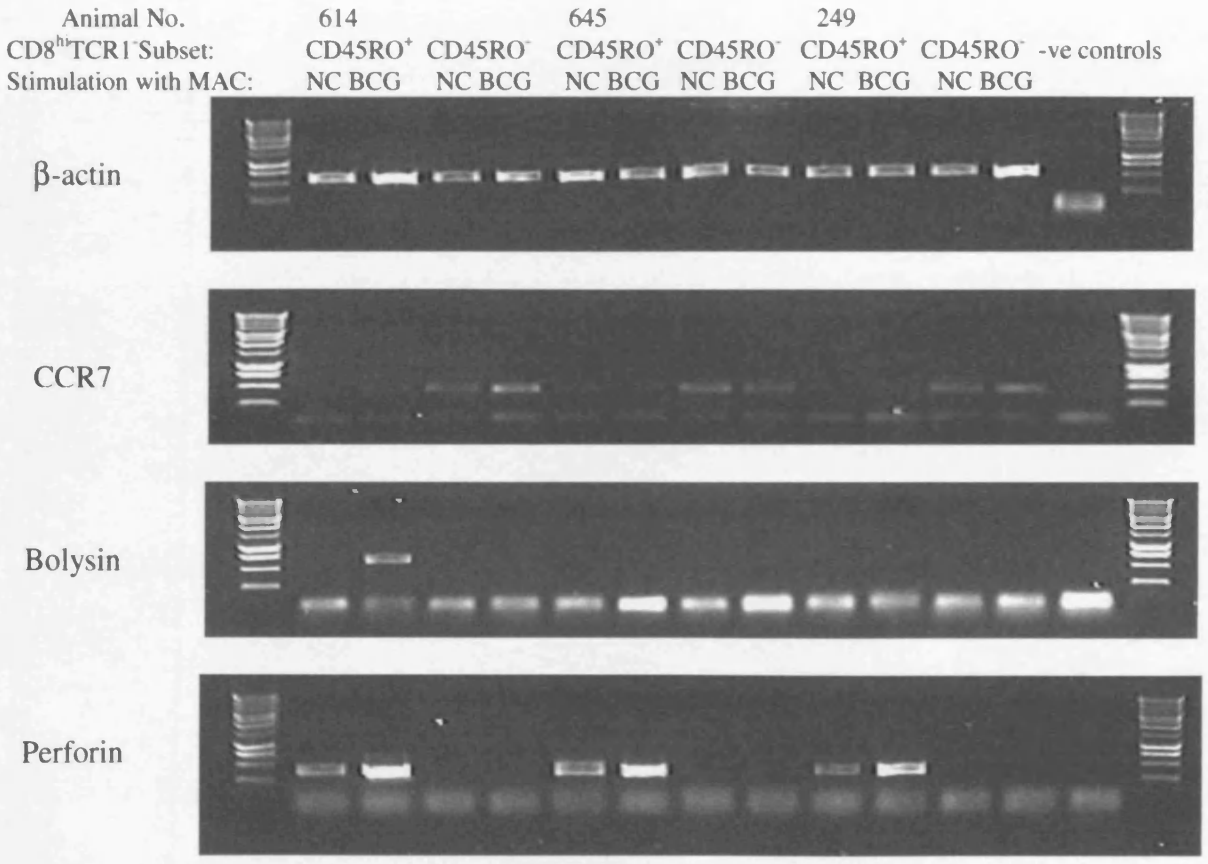


Figure 5.3.4 RT-PCR analysis of expression of CCR7, Bolysin and perforin by $CD8^+$ T cells from BCG-vaccinated animals after culture with mycobacteria. $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$ T cells were sorted from blood from three BCG-vaccinated animals (614, 645 and 249) as described previously and cultured in vitro with uninfected and BCG-infected Mϕ for 5 days. Cells were then lysed with GTC and RNA was extracted using Qiagen RNAsy kit. Samples were treated with DNAses and cDNA was synthesised from the RNA as described in the materials and methods. Standard PCR was then carried to investigate expression of the following β-actin, CCR7, Bolysin and perforin in the samples.

Figure 5.3.4 shows a composition of agarose gels containing the electrophorised PCR products for β-actin, CCR7, granulysin and perforin. It was found that β-actin was expressed equally by both $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells from all three animals. CCR7 was found to be expressed mainly by the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells. However, faint bands corresponding to CCR7 were also visible in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animals 614 and 645 suggesting the presence of either a contaminating population of naive T cells or a population of central memory T cells. Expression of granulysin was detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from only animal 614 and expression of this molecule required stimulation with BCG-infected Mϕ (figure 5.3.4). Perforin was found to be expressed in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from all three animals. The bands representing perforin were stronger and brighter in the $CD8^{hi}TCR1^{-}CD45RO^{+}$T cells that had been cultured with BCG-infected Mϕ compared to those cultured with uninfected Mϕ (figure 5.3.4).

These results suggest that the two cytolytic molecules perforin and granulysin, although proposed to act together, are differentially expressed as only in one animal 614 were both of these molecules expressed whereas animals 645 and 249 expressed only perforin.

5.3.3 Analysis of surface phenotype and expression of perforin by mycobacteria-reactive $CD8^{hi}TCR1^{-}$ T cells

It was demonstrated in chapter 4 by flow cytometry that the cytotoxic molecule perforin was expressed predominantly by $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells. In addition, the analysis of perforin expression by RT-PCR in mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells suggested that expression of perforin is up-regulated after culture with BCG-infected Mϕ. To corroborate these findings, flow cytometry was used to analyse surface phenotype and expression of perforin by $CD8^{hi}TCR1^{-}$T cells from the three BCG-vaccinated animals on the same day after culture with uninfected or BCG-infected Mϕ for 5 days.

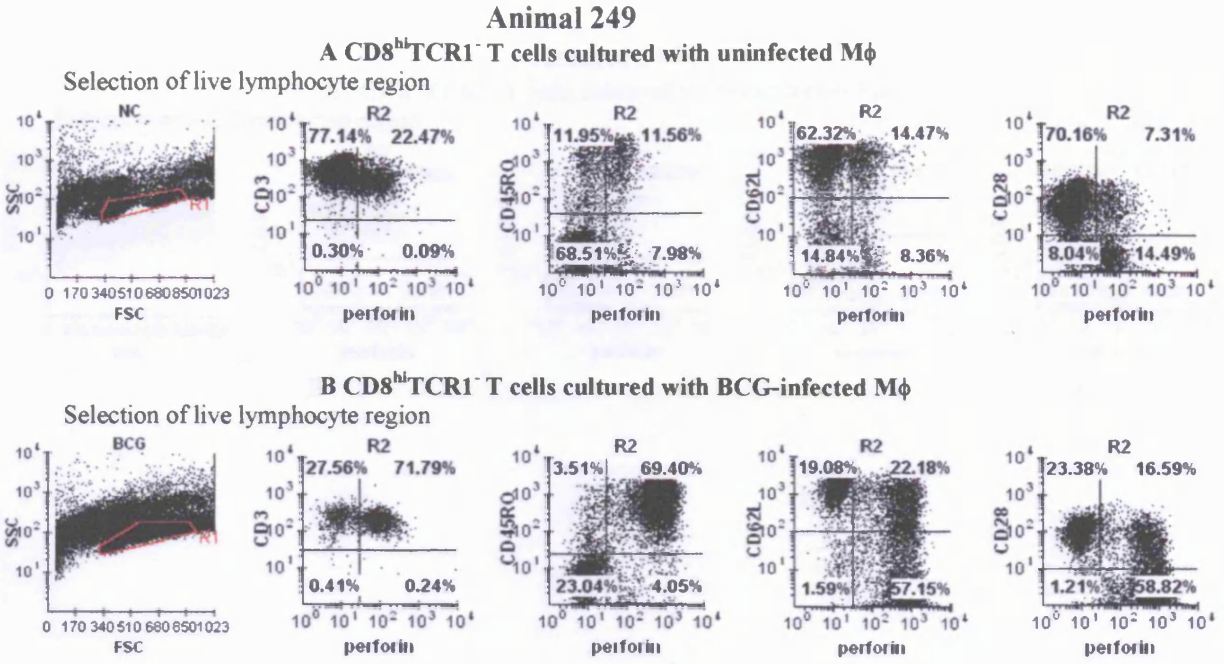


Figure 5.3.5. Expression of perforin by $CD8^{hi}TCR1^-$ T cells from a BCG vaccinated animal (249) after culture with BCG-infected Mϕ. PBMC were isolated from blood taken at 14 wks post-BCG vaccination. $CD8^+$ cells were sorted from the PBMC using the MACS paramagnetic system and were stained with an antibody to the γδ T cell receptor (TCR1). $CD8^{hi}TCR1^-$ T cells were then sorted from the $CD8^+$ cells on a Moflo cell sorter and stimulated for 5 days with uninfected (A) or BCG-infected (B) Mϕ. Cells were then washed and stained with antibodies to CD8, TCR1 and one of the following CD3, CD45RO, CD62L and CD28. Cells were fixed, permeabilised and stained with an antibody to perforin. Resting and activated lymphocytes were gated as shown (R1) and analysed for expression of perforin and surface molecules.

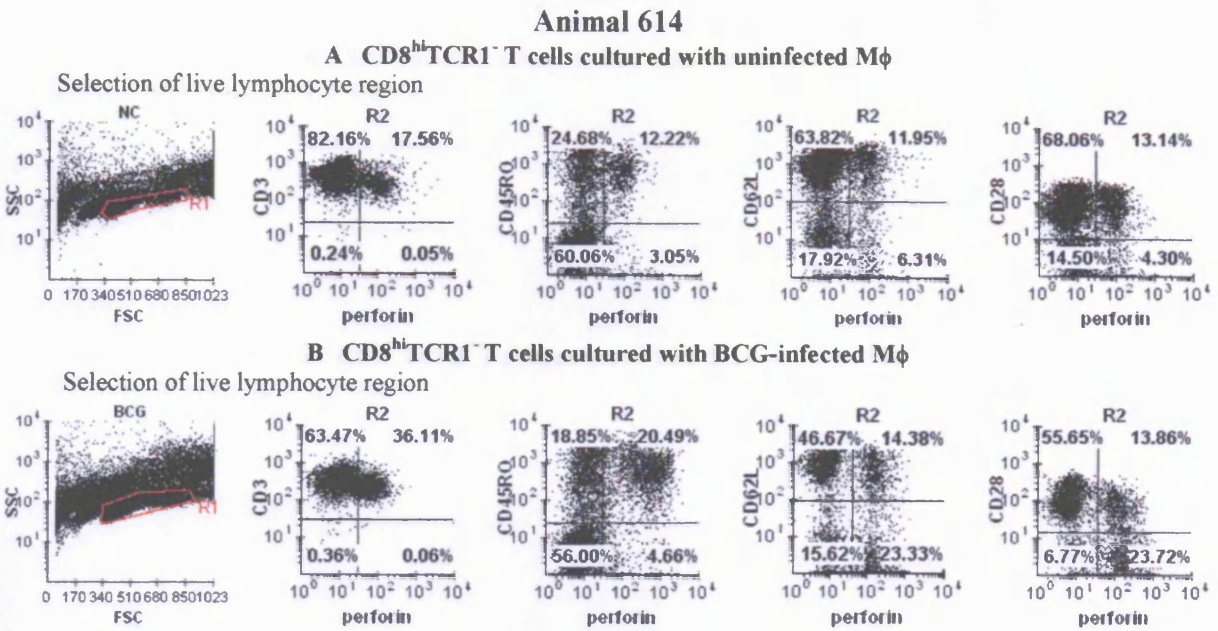


Fig 5.3.6 Expression of perforin by $CD8^{hi}TCR1^-$ T cells from a BCG vaccinated animal (614) after culture with BCG-infected Mϕ. PBMC were isolated from blood taken at 14 wks post-BCG vaccination. $CD8^+$ cells were sorted from the PBMC using the MACS paramagnetic system and were stained with an antibody to the γδ T cell receptor (TCR1). $CD8^{hi}TCR1^-$ T cells were then sorted from the $CD8^+$ cells on a Moflo cell sorter and stimulated for 5 days with uninfected (A) or BCG-infected (B) macrophages. Cells were then washed and stained with antibodies to CD8, TCR1 and one of the following CD3, CD45RO, CD62L and CD28. Cells were fixed, permeabilised and stained with an antibody to perforin. Resting and activated lymphocytes were gated as shown (R1) and analysed for expression of perforin and surface molecules.

Animal 645

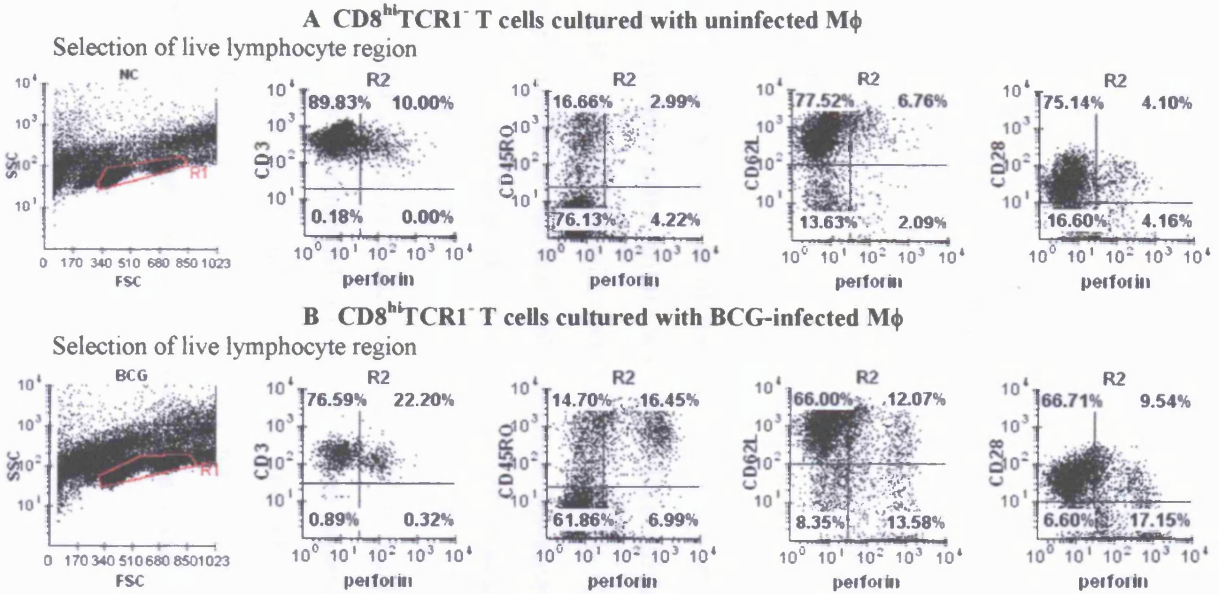


Figure 5.3.7 Expression of perforin by $CD8^{hi}TCR1^-$ T cells from a BCG vaccinated animal (645) after culture with BCG-infected Mϕ. PBMC were isolated from blood taken at 14 wks post-BCG vaccination. $CD8^+$ cells were sorted from the PBMC using the MACS paramagnetic system and were stained with an antibody to the γδ T cell receptor (TCR1). $CD8^{hi}TCR1^-$ T cells were then sorted from the $CD8^+$ cells on a Moflo cell sorter and stimulated for 5 days with uninfected (A) or BCG-infected (B) Mϕ. Cells were then washed and stained with antibodies to CD8, TCR1 and one of the following CD3, CD45RO, CD62L and CD28. Cells were fixed, permeabilised and stained with an antibody to perforin. Resting and activated lymphocytes were gated as shown (R1) and analysed for expression of perforin and surface molecules.

No difference was found in the percentage of $CD8^{hi}TCR1^-$ T cells expressing perforin before culture and after culture with uninfected Mϕ. Figure 5.3.5 shows that in animal 249, a higher percentage of $CD8^{hi}TCR1^-$ T cells expressed perforin after culture with BCG-infected Mϕ compared to uninfected Mϕ. These $CD8^{hi}TCR1^-Perforin^+$ T cells expressed CD45RO and CD3 but predominantly lacked expression of CD62L and CD28. However, smaller increases were also observed in the percentage of $CD8^{hi}TCR1^-Perforin^+$ T cells from animal 249 that expressed CD62L and CD28.

Similarly in animal 614, the percentage of $CD8^{hi}TCR1^-$ T cells that express perforin increased after culture with BCG-infected Mϕ compared to cells cultured with Mϕ. The $CD8^{hi}TCR1^-Perforin^+$ T cells from animal 614 were $CD45RO^+$, $CD3^+$, $CD62L^-$ and $CD28^-$ (Figure 5.3.6).

Figure 5.3.7 shows that an increased percentage of $CD8^{hi}TCR1^-$ T cells from animal 645 expressed perforin after culture with BCG-infected Mϕ compared to uninfected Mϕ. These $CD8^{hi}TCR1^-perforin^+$ T cells were predominantly $CD45RO^+$, $CD3^+$, $CD62L^-$ and $CD28^-$. However, similar to animal 249, smaller increases in the percentage of $CD8^{hi}TCR1^-perforin^+$ T cells that were $CD62L^+$ and $CD28^+$ was also observed (Fig 5.3.7).

In animal 614 and 645 the increases in $CD8^{hi}TCR1^-Perforin^+$ T cells after stimulation with BCG were less pronounced compared to that observed in animal 249. This is in contrast with the proliferative and IFN-γ data, in which mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells from animals 614 and 645 proliferated to a greater extent and produced more IFN-γ than the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells from animal 249.

5.3.4 Cytotoxic ability of mycobacteria-reactive $CD8^+$ T cells induced by BCG vaccination

It is long-established that T cells play a central role in protection against infection with mycobacteria, but the mechanisms by which T cells contribute to the control mycobacteria replication are poorly understood. One mechanism by which T cells may restrict the growth of mycobacteria within Mϕ is through direct killing of the mycobacteria and the Mϕ. An alternative mechanism would involve the killing of the infected Mϕ and the subsequent release of the mycobacteria allowing it to be taken-up and killed by an uninfected activated Mϕ (Fratazzi, Arbeit et al. 1999). There are two main pathways of T cell-mediated killing, the exocytosis of cytotoxic granules and ligation of CD95 to CD95L (Russell and Ley 2002).

The results of this study show that mycobacteria-reactive BCG-reactive $CD8^{hi}TCR1^-$ T cells develop after BCG-vaccination. In response to culture with BCG-infected Mϕ these cells up-regulate expression of perforin and in one animal these cells also expressed Bolysin. To investigate the ability of these cells to kill BCG-infected Mϕ, $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$ T cells were isolated from three BCG-vaccinated animals one the same day and three naïve animals as described in the materials and methods. These cells were cultured with BCG-infected Mϕ for 5 days, before being cultured with freshly isolated uninfected or BCG-infected Mϕ labelled with ^{51}Cr. After 4.5 hrs the release of ^{51}Cr into supernatants was measured and the percentage of specific lysis was determined as described in the materials and methods.

Figure 5.3.8 shows that the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells from BCG-vaccinated animals 614 and 645 specifically lysed BCG-infected but not uninfected Mϕ. No cytotoxic activity was detected in the $CD8^{hi}TCR1^-CD45RO^-$ T cells from any of the BCG-vaccinated animals. This is consistent with the previous findings in which the $CD8^{hi}TCR1^-CD45RO^-$T cells did not proliferate or produce IFN-γ after stimulation with BCG-infected Mϕ or DC.

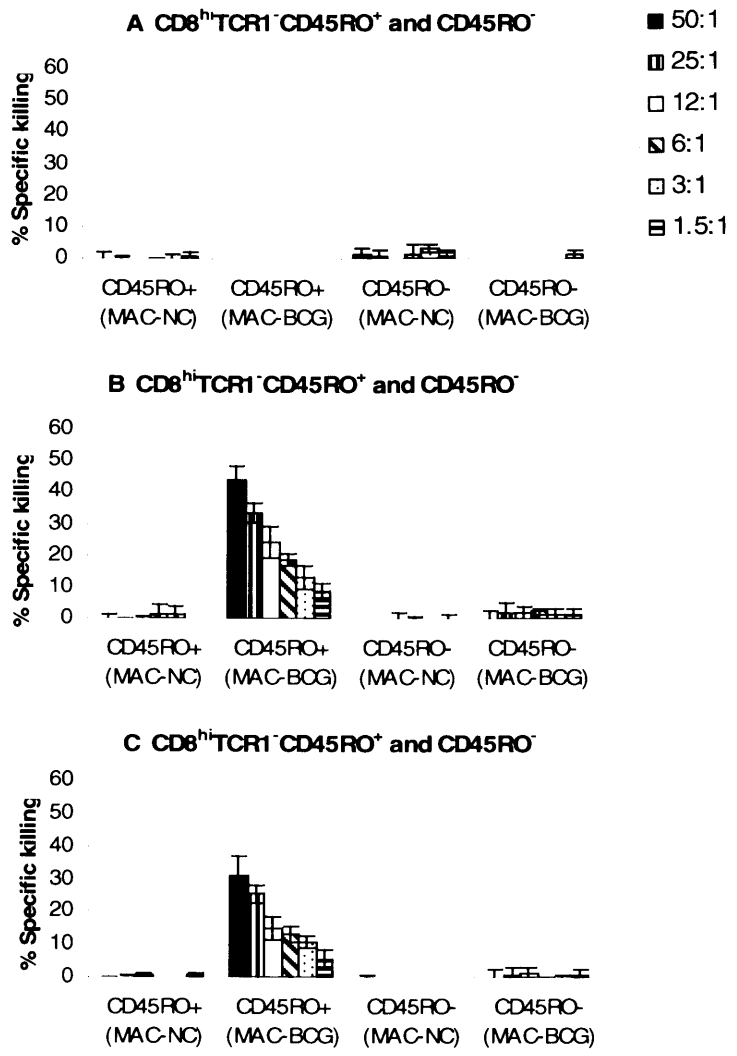


Figure 5.3.8 Killing of BCG-infected Mϕ by $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from three BCG-vaccinated animals 249 (A), 614 (B) and 645 (C). $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells were sorted from blood from BCG-vaccinated animals as described previously. Cells were stimulated with BCG-infected Mϕ for 5 days. Freshly prepared BCG-infected (MAC-BCG) and uninfected Mϕ (MAC-NC) were labelled with ^{51}Cr and were added to the $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells at different effector to target ratios for 4.5 hrs. Supernatants were then removed and the release of ^{51}Cr by the labelled Mϕ was determined and the percentage killing was calculated as described in the materials and methods. The mean and standard deviation of triplicate samples is shown.

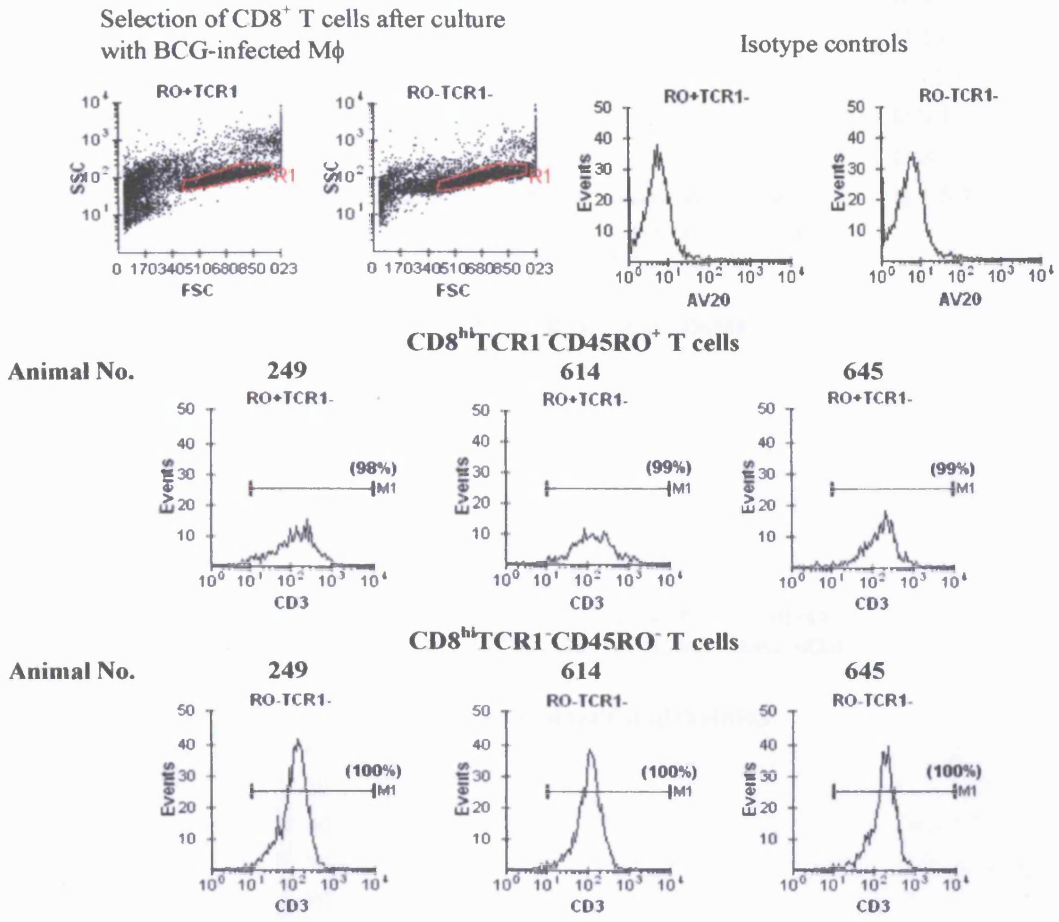


Figure 5.3.9 Expression of CD3 by $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells after culture with BCG-infected Mϕ. $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells was isolated from the three BCG-vaccinated animals and cultured for 5 days with BCG-infected Mϕ. The cells were then washed and stained with an antibody to CD3. Expression of the isotype control AV20 was analysed in all three animals was found to be negative.

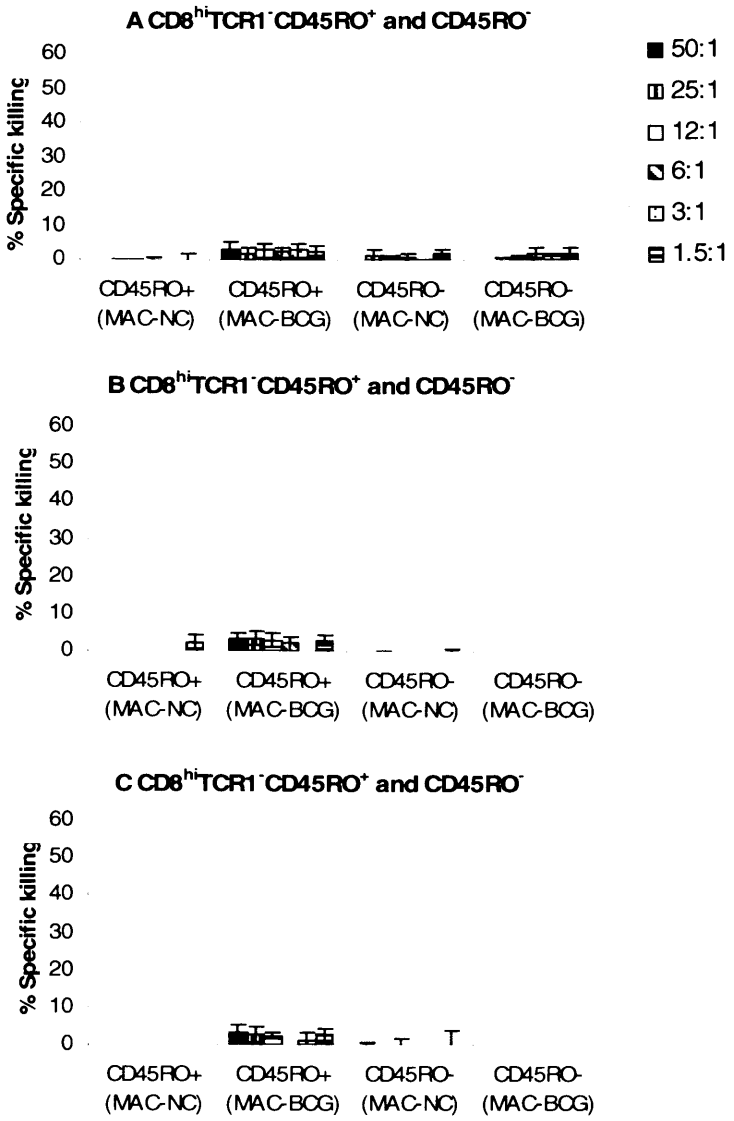


Figure 5.3.10 Killing of BCG-infected Mϕ by $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from age-matched nonvaccinated animals 370 (A), 372 (B) and 383 (C). $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells were sorted from blood from BCG-vaccinated animals as described previously. Cells were stimulated with BCG-infected Mϕ for 5 days. Freshly prepared BCG infected (MAC-BCG) and uninfected Mϕ (MAC-NC) were labelled with ^{51}Cr and were added to the $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells at different effector to target ratios for 4.5 hrs. The amount of ^{51}Cr released into the supernatant by the labelled Mϕ was determined and the percentage killing was calculated as described in the materials and methods. The mean and standard deviation of triplicate samples are shown.

Suprisingly, no cytolytic activity was detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animal 249 as these cells proliferated, produced IFN-γ and up-regulated perforin expression after culture with BCG-infected Mϕ. One possibility is that the mycobacteria-reactive immune response induced by BCG-vaccination had declined in this animal when the CTL assay was performed. It is also possible that the 5-day culture period with BCG-infected Mϕ may not be the optimal length of time for the mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells in animal 249 to exhibit cytolytic activity.

To show that the $CD8^{hi}TCR1^{-}$ T cells used in the ^{51}Cr release were not NK cells, the $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells were analysed for expression of CD3 after the 5 day culture with BCG-infected Mϕ and just prior to the cytotoxicity assay. Both subsets were found to be 98-100% positive for CD3, therefore it is unlikely that the observed killing was due to a population of contaminating NK cells (Fig 5.3.9).

In order to show that the ^{51}Cr release assay was detecting antigen-specific killing, the ability of $CD8^{+}$ T cells from three non-vaccinated age-matched animals to kill BCG-infected Mϕ was investigated. Figure 5.3.10 shows that no cytotoxic activity was detected in either the $CD8^{hi}TCR1^{-}CD45RO^{+}$ or the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells from the non-vaccinated animals.

5.4 Development of immune responses after BCG vaccination of neonatal animals

5.4.1 Analysis of mycobacteria-reactive $CD8^{hi}TCR1^{-}$ T cells in neonatal animals after BCG-vaccination

The above experiments demonstrated that mycobacteria-reactive $CD8^{+}$ T cells are detected in animals vaccinated with BCG at 6 mths of age. It has been proposed that cattle should be vaccinated with BCG as neonates prior to the animals becoming sensitised by exposure to environmental mycobacteria such as *M. avium*. In mice, reactivity to environmental mycobacteria has been shown to reduce the ability of BCG to induce a strong immune response (Brandt, Feino Cunha et al. 2002). Similarly in cattle, this has also been shown to decrease the efficacy of BCG vaccination at conferring protection against TB in cattle (Buddle, Wards et al. 2002). Furthermore exposure to *M. avium*, prior to vaccination with

BCG has been shown to bias the immune response towards antigens shared between the BCG and *M. avium* (Howard, Kwong et al. 2002).

To investigate whether mycobacteria-reactive $CD8^+$ T cells are induced by BCG vaccination of neonatal animals, $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$T cells were isolated from blood, before and at intervals after vaccination with BCG. These two T cell subsets, total $CD8^+$ cells and PBMC were cultured with uninfected, BCG-infected or *M. avium*-infected Mϕ for 5 days. Mycobacteria-reactive T cells were detected by proliferation measured using ^3H TdR incorporation and production of IFN-γ measured by ELISA.

Prior to vaccination strong responses to mycobacteria were detected in the $CD8^{hi}TCR1^-CD45RO^+$ T cells from animals 29 and 23. High background responses were also observed to mycobacteria in the total $CD8^+$ cells from all three animals and in the PBMC from animals 23 and 34. The mechanisms behind these responses prior to vaccination are unclear but they appear to require the presence of mycobacteria.

In animal 23, prior to vaccination and also at 3 wks post-vaccination the $CD8^{hi}TCR1^-CD45RO^+$ T cells from animal 23 responded more strongly to *M. avium*-infected Mϕ compared to BCG-infected Mϕ. An increased level of proliferation and IFN-γ production by $CD8^{hi}TCR1^-CD45RO^+$ T cells to BCG-infected Mϕ was detected at 9 wks post-vaccination. This response was greater than that observed prior to vaccination suggesting the presence of vaccine induced BCG-reactive T cells (Fig 5.4.1A). In addition, at this timepoint the $CD8^{hi}TCR1^-CD45RO^+$ T cells responded more highly to BCG-infected Mϕ compared to *M. avium*-infected Mϕ (Fig 5.4.1A).

In animal 23, at week 3 post-vaccination, a transient low level response to mycobacteria was detected in the $CD8^{hi}TCR1^-CD45RO^-$ T cells after culture *M. avium*-infected Mϕ (Fig 5.4.1B). After vaccination with BCG, the proliferative response after culture of the total $CD8^+$ cells from animal 23 with mycobacteria declined but the production of IFN-γ remained high. A response to uninfected Mϕ was detected in the total $CD8^+$ cells at 5 and 9 wks, suggesting that the response to mycobacteria detected at these weeks may not have been antigen-specific (Fig 5.4.1C). It is thought that this response to uninfected Mϕ may also reflect the heterogeneous nature of this population as it is unclear what cell types is responding.

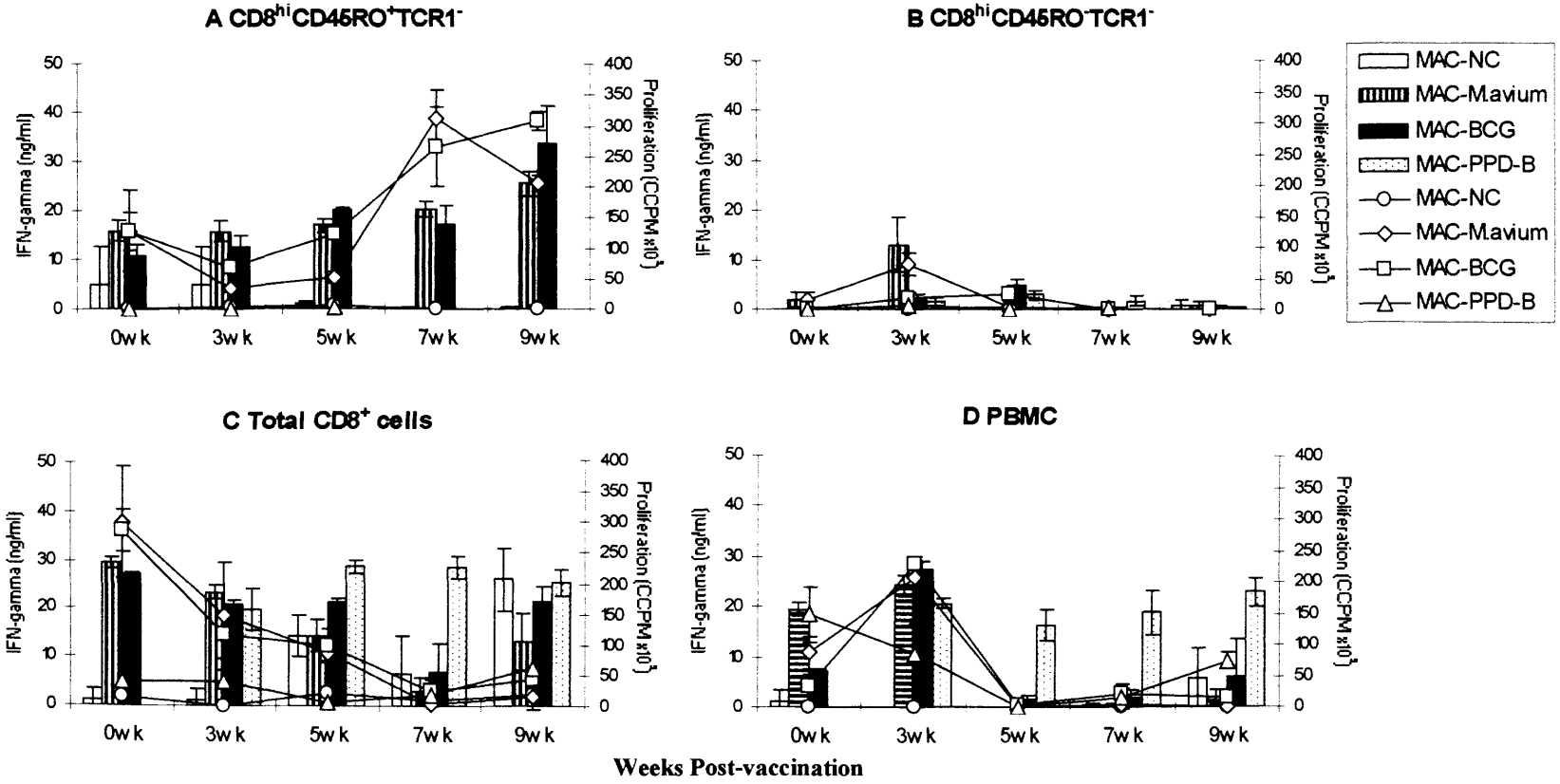


Figure 5.4.1 BCG vaccination of a neonate (23) induces $CD8^+$ T cells and PBMC to proliferate and produce IFN-γ after culture with mycobacteria. $CD8^+$ cells were isolated from PBMC using MACS isolation system. $CD8^+$ T cells were then stained with antibody to the γδ TCR (TCR1) and CD45RO before being sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The two MoFlo-sorted subsets $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected, PPD-B, *M. avium*- or BCG-infected Mϕ (MAC). Proliferation measured by incorporation of ^3H TdR and is shown as CCPM (line). Production of IFN-γ was measured by ELISA and is shown as ng/ml (column). The mean and standard deviation are shown for triplicate samples from one BCG-vaccinated animal (23).

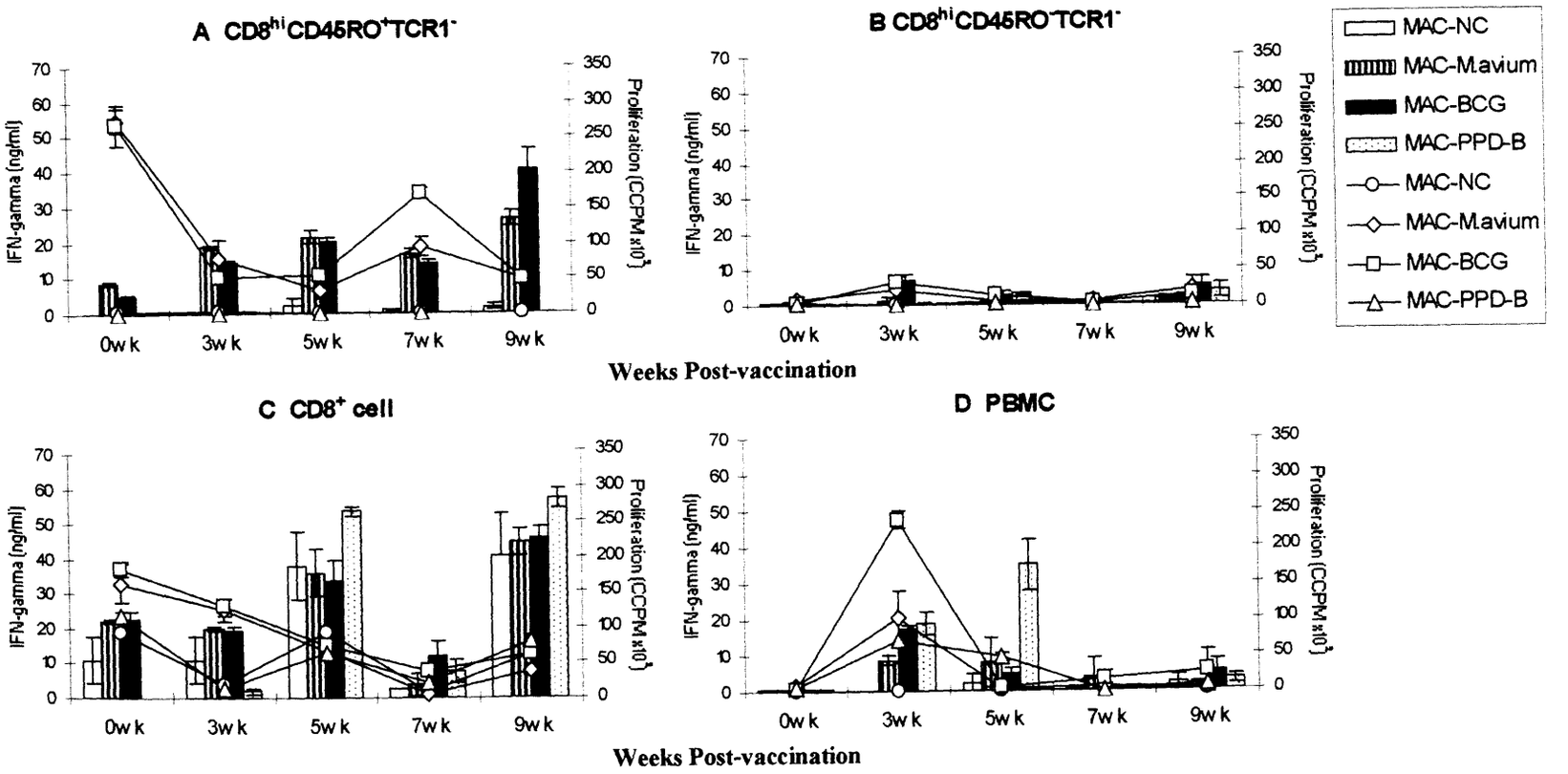


Figure 5.4.2 BCG vaccination of a neonate (29) induces $CD8^+$ T cells and PBMC to proliferate and produce IFN-γ after culture with mycobacteria. $CD8^+$ cells were isolated from PBMC using MACS isolation system. $CD8^+$ T cells were then stained with antibody to the γδ TCR (TCR1) and CD45RO before being sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The two MoFlo-sorted subsets $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected, PPD-B, *M. avium*- or BCG-infected Mϕ (MAC). Proliferation measured by incorporation of ^3H TdR and is shown as CCPM (line). Production of IFN-γ was measured by ELISA and is shown as ng/ml (column). The mean and standard deviation are shown for triplicate samples from one BCG-vaccinated animal (29)

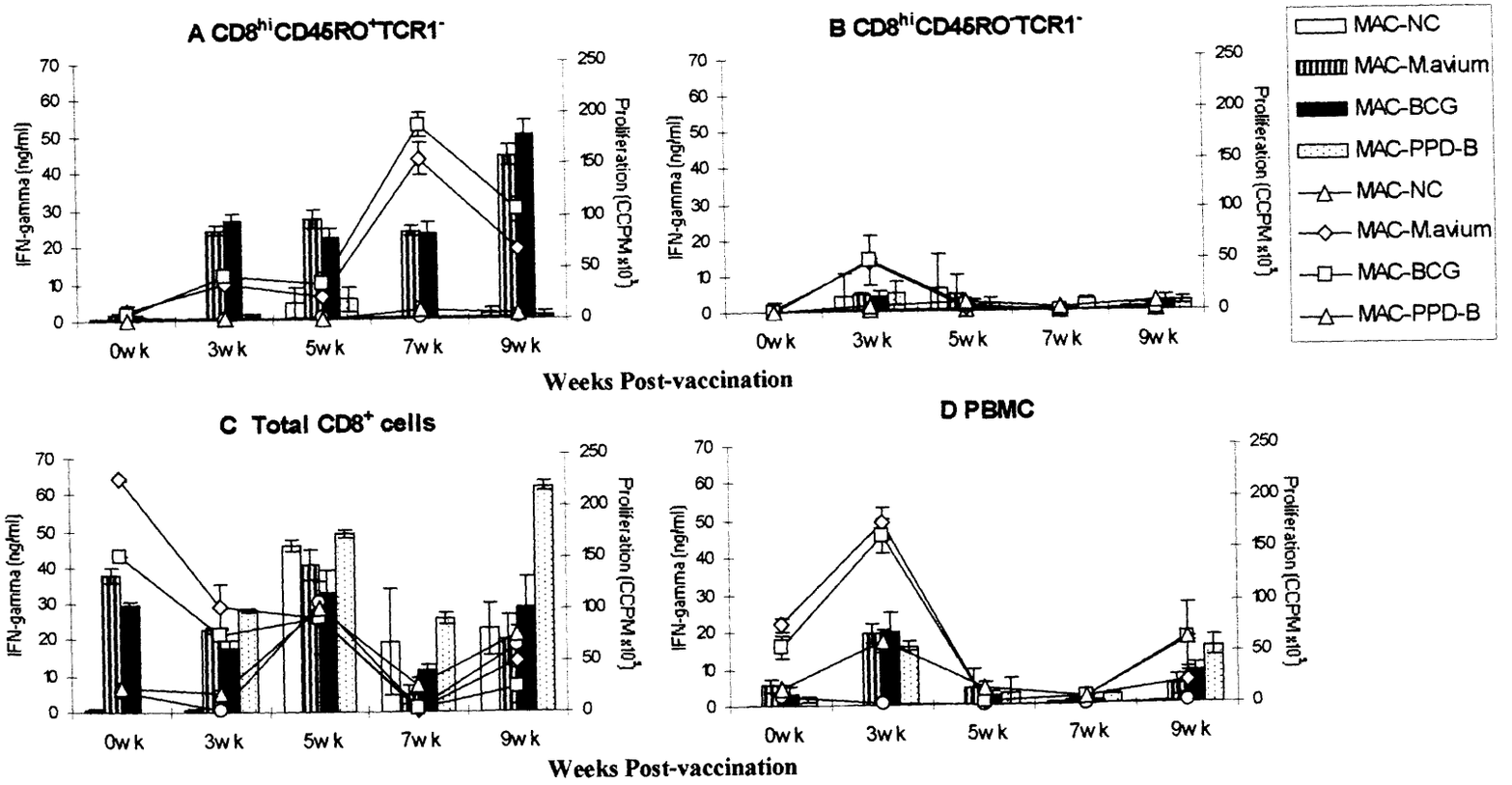


Figure 5.4.3 BCG vaccination of a neonatal animal (34) induces $CD8^+$ T cells and PBMC to proliferate and produce IFN-γ after culture with mycobacteria. $CD8^+$ cells were isolated from PBMC using MACS isolation system. $CD8^+$ T cells were then stained with antibody to the γδ TCR (TCR1) and CD45RO before being sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The two MoFlo-sorted subsets $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected, PPD-B, *M. avium*- or BCG-infected Mϕ (MAC). Proliferation measured by incorporation of ^3H TdR and is shown as CCPM (line). Production of IFN-γ was measured by ELISA and is shown as ng/ml (column). The mean and standard deviation are shown for triplicate samples from one BCG-vaccinated animal (34).

Vaccination with BCG, induced the development of PPD-B-reactive $CD8^+$ cells as the response to PPD-B in the total $CD8^+$ cells increased post-vaccination (Fig 5.4.1C).
The PBMC from animal 23 responded highly to *M. avium* prior to BCG vaccination and also at 3 wks post-vaccination but this response declined by 5 wks post-vaccination (Fig 5.4.1D). At 3 wks post-vaccination, the level of response of the PBMC to BCG-infected Mϕ had increased significantly compared to before vaccination. After vaccination with BCG, cells in the PBMC also produced high levels of IFN-γ after culture with PPD-B. This response to PPD-B was first detected at 3 wks post-vaccination and was detected until the end of the experiment, suggesting that BCG-vaccination induced a memory $CD4^+$ T cell response in animal 23 (Fig 5.4.1D).

In animal 29, the responses to mycobacteria detected in the $CD8^{hi}TCR1^-CD45RO^+T$ cells before vaccination declined after vaccination with BCG. Mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+T$ cells could be detected in blood again at 7 wk post-vaccination and which point these cells proliferated more highly to BCG-infected Mϕ than to *M. avium*-infected Mϕ (Fig 5.4.2A). The level of production of IFN-γ by the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+T$ cells after culture with both BCG- and *M. avium*-infected Mϕ also increased post-vaccination is shown to peak at 9 wks post-vaccination. At this timepoint the $CD8^{hi}TCR1^-CD45RO^+$ T cells produced more IFN-γ after culture with BCG compared to *M. avium* (Fig 5.4.2A).
The total $CD8^+$ cells from animal 29 responded strongly prior to vaccination after culture with uninfected and infected Mϕ. The proliferative response of these cells declined post-vaccination whereas the amount of IFN-γ produced in response to uninfected and infected Mϕ increased post-vaccination. The observed response appears to be antigen independent as the total $CD8^+$ cells respond highly to both infected and uninfected Mϕ (Fig 5.4.2C).
No response prior to vaccination was detected in the PBMC from animal 29. However, 3 wks after vaccination with BCG, these cells proliferated after culture with PPD-B, BCG-infected and *M. avium*-infected Mϕ. This vaccine-induced response in the PBMC was short-lived as these cells did not respond after 5 wks post-vaccination. (Fig 5.4.2D).

In contrast with animals 23 and 29, the $CD8^{hi}TCR1^-CD45RO^+$ T cells from animal 34 did not respond to mycobacteria prior to vaccination. After BCG vaccination, the $CD8^{hi}TCR1^-$

$CD45RO^+$ T cells proliferated and produced IFN-γ in response to both BCG-infected and *M. avium*-infected Mϕ. The level of proliferation observed by the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells peaked at 7 wks post-vaccination whereas highest production of IFN-γ was detected at 9 wks post-vaccination. (Fig 5.4.3A).
Consistent with animals 23 and 29, in animal 34 the $CD8^{hi}TCR1^-CD45RO^-$ T cells proliferated at 3 wks post-vaccination after culture with BCG-infected Mϕ (Fig 5.4.3B).

After vaccination with BCG the strong response to mycobacteria detected in the total $CD8^+$ cells from animal 34 prior to vaccination declined. However, the level of response of the total $CD8^+$ cells to PPD-B increased post-vaccination. In addition, at 5, 7 and 9 wks post-vaccination, the $CD8^+$ cells responded highly to uninfected Mϕ (Fig 5.4.3C).

The PBMC from animal 34 are shown in figure 5.4.43D to proliferate after culture with BCG and *M. avium*-infected Mϕ prior to vaccination. At 3 wks post-vaccination the level of the response had increased significantly both in terms of proliferation and IFN-γ production. Also at this time-point the PBMC responded to PPD-B. After 3 wks post-vaccination, no mycobacteria-reactive cells were detected in the PBMC until a low level response was detected at 9 wks post-vaccination, suggesting that the BCG vaccine was cleared quickly and may have only induced a weak memory response (Fig 5.4.3D).

In order to demonstrate that the responding $CD8^+$ T cells were not NK cells that have been described in neonates by Hope et al, the $CD8^{hi}TCR1^-CD45RO^+$, $CD8^{hi}TCR1^-CD45RO^-$ T cells from the neonatal animals were analysed for expression of CD3 after culture with BCG-infected Mϕ (Hope, Sopp et al. 2002). The total $CD8^+$ cell population was also analysed for expression of CD3 as this population of cells has been used in past studies that have investigated $CD8^+$ T cell responses in cattle.
The $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$ T cells from the three animals are shown in figure 5.4.4 to uniformly express CD3 after culture with BCG-infected Mϕ. In contrast, a population of $CD3^-$ cells were observed in the total $CD8^+$ cells from all three animals which suggest that this population of cells contains some NK cells.

5.4.2 Analysis of the ability of BCG-reactive $CD8^{hi}TCR1^-$ T cells from neonatal animals to kill BCG-infected cells

To investigate whether the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells detected in the BCG vaccinated neonatal animals can kill BCG-infected Mϕ, the $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$ T cells were isolated and cultured as described previously and analysed for cytotoxicity in a ^{51}Cr release assay.

No cytolytic activity was detected in either the $CD8^{hi}TCR1^-CD45RO^+$ or $CD8^{hi}TCR1^-CD45RO^-$ T cells from animal 23. The $CD8^{hi}TCR1^-CD45RO^+$ T cells from animals 29 and 34 were found to specifically kill BCG-infected Mϕ demonstrating approximately 35% and 70% specific lysis at an effector to target ratio of 50:1, respectively. The observed killing titrated out but was still detected at lower E:T ratios (Fig. 5.4.5). The $CD8^{hi}TCR1^-CD45RO^+$and $CD8^{hi}TCR1^-CD45RO^-$ T cells were shown to be $CD3^+$ prior to being used in the ^{51}Cr release assay (Fig 5.4.4). Thus, any killing observed is likely to be due to $CD8^+\alpha\beta$ T cells.

The chromium release assay was also performed using $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$ T cells from two age-matched unvaccinated controls. No specific killing was observed in either of the two control animals suggesting that the killing observed in the vaccinated animals is antigen specific (Fig 5.4.6).

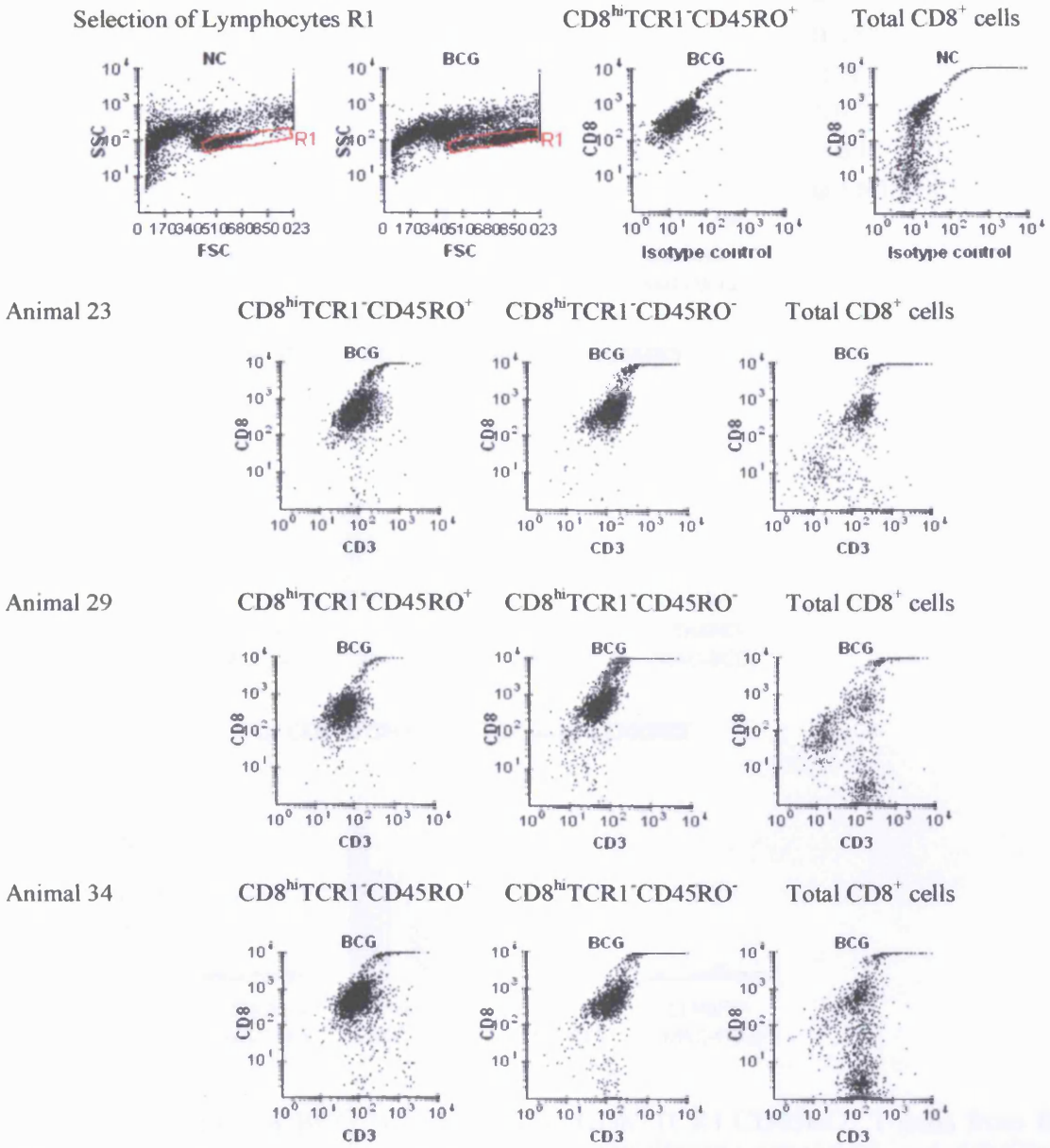


Figure 5.4.4 Cell surface expression of CD3 by sorted $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells, $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells and total $CD8^{+}$ cells after culture with BCG. PBMC was isolated from blood from the three BCG vaccinated neonatal animals (23, 29 and 34) and total $CD8^{+}$ cells were isolated using MACS system. $CD8^{+}$ cells were stained with antibodies to CD45RO and the γδ T cell receptor. The $CD8^{hi}$ expressing cells were selected and sorted on a Moflo cell sorter based upon expression of TCR1 and CD45RO to give $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells. The isolated $CD8^{+}$ cells and $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells were cultured with BCG-infected Mϕ for 5 days and then harvested and analysed for expression of CD3 using flow cytometry.

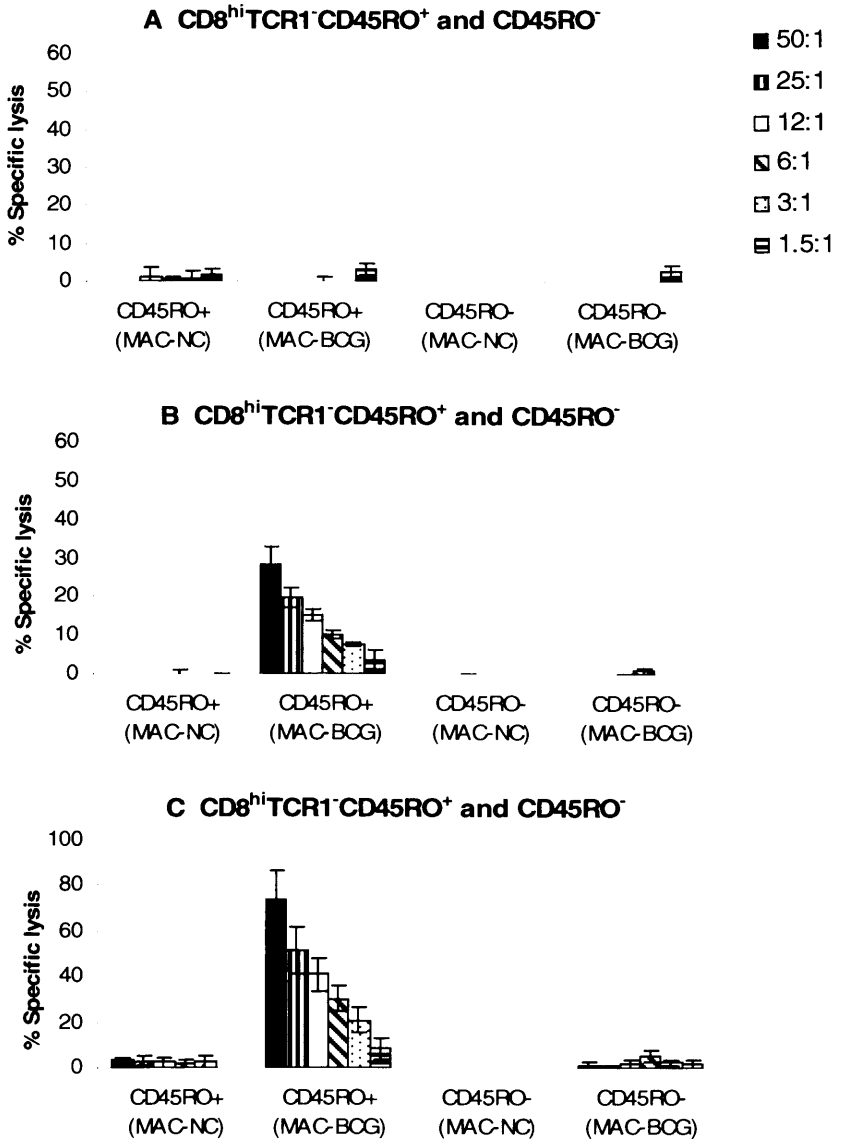


Figure 5.4.5 Killing of BCG-infected Mϕ by $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from BCG-vaccinated neonates 23 (A), 29 (B), 34 (C). $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells were sorted from blood as described previously. Cells were stimulated with BCG-infected Mϕ for 5 days. Freshly prepared BCG infected (MAC-BCG) and uninfected Mϕ (MAC-NC) were labelled with ^{51}Cr and added to the $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells at different effector to target ratios for 4.5 hrs. The level of ^{51}Cr released by the labelled Mϕ into the supernatant was measured and the percentage killing was calculated as described in the materials and methods. The mean and standard deviation of triplicate samples from one experiment are shown and are representative of two experiments.

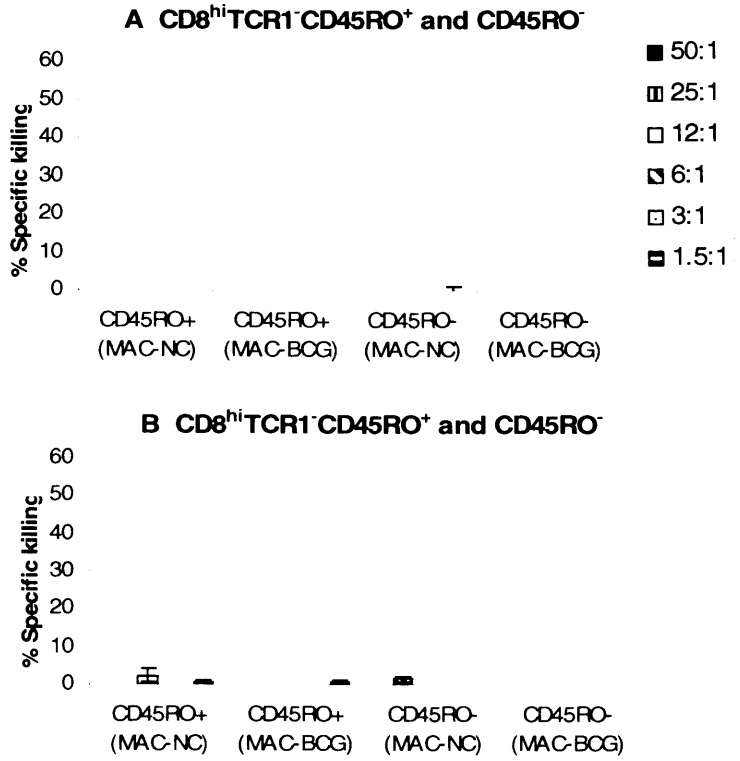


Figure 5.4.6 Killing of BCG-infected Mϕ by $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from age-matched non-vaccinated neonates 12 (A) and 14 (B). $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells were sorted from blood from two age-matched naïve animals as described previously. Cells were stimulated with BCG-infected Mϕ for 5 days. Freshly prepared BCG infected (MAC-BCG) and uninfected Mϕ (MAC-NC) were labelled with ^{51}Cr and added to the $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells at different effector to target ratios for 4.5 hrs. The level of ^{51}Cr released by the labelled Mϕ into the supernatant was measured and the percentage killing was calculated as described in the materials and methods. The mean and standard deviation of triplicate samples are shown.

5.5 Heterologous prime-boost strategy involving priming with VV-Ag85A and boosting with BCG

5.5.1 Analysis of immune responses induced by vaccination with VV-Ag85A and boosting with BCG

At present no other TB vaccine has been shown to confer a greater level of protection against TB than BCG. However, greater levels of protection have been achieved in animal models with heterologous-prime boost strategies that aim to enhance the immune response induced by BCG. A number of recombinant viral vectors expressing mycobacterial antigens have been shown to induce strong immune responses when used in combination with BCG. The efficacy and safety of MVA-Ag85A at boosting BCG-induced immune response is currently being evaluated in phase II clinical trials (McShane, Pathan et al. 2005).

The aim of this experiment was to induce a strong $CD8^+$ T cell response to Ag85 by priming animals with recombinant virulent vaccinia virus expressing Ag85 (VV-Ag85) and boosting with BCG at 8 wks post-VV-Ag85A. The VV-Ag85 was chosen to be administered first as at the time of the experiment it was unclear if vaccination with BCG induced a $CD8^{hi}TCR1^-$ T cell response. The VV replicates in the cytoplasm of bovine cells and thus allowing the Ag85 to gain access to the MHC class I processing pathway. Also, studies in humans have shown that $CD8^+$ T cells from *M. tuberculosis*-infected and BCG-vaccinated individuals respond to Ag85 peptides. $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$ T cells were isolated before and at intervals after vaccination with the priming vaccineVV-Ag85A and booster vaccine BCG. These two sort subsets, total $CD8^+$ and PBMC were cultured with uninfected, MVA-WT, MVA-Ag85 and BCG-infected Mϕ for 5 days. Mycobacteria-reactive T cells were detected by proliferation measured using ^3H TdR incorporation and production of IFN-γ measured by ELISA.

Two weeks after vaccination with VV-Ag85A, mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells were detected from animal 697, as shown by a response to BCG-infected Mϕ (Fig 5.5.1A). The responding $CD8^{hi}TCR1^-CD45RO^+$ T cells proliferated and produced IFN-γ. Vaccination with BCG at 8 wks post-VV-Ag85 boosted the response of the $CD8^{hi}TCR1^-CD45RO^+$ T cells. However, this boosting effect was transient as no response to mycobacteria was detected in the $CD8^{hi}TCR1^-CD45RO^+$T cells at wks 12 and 14 post-VV-Ag85 (4 and 6 wks post-BCG).

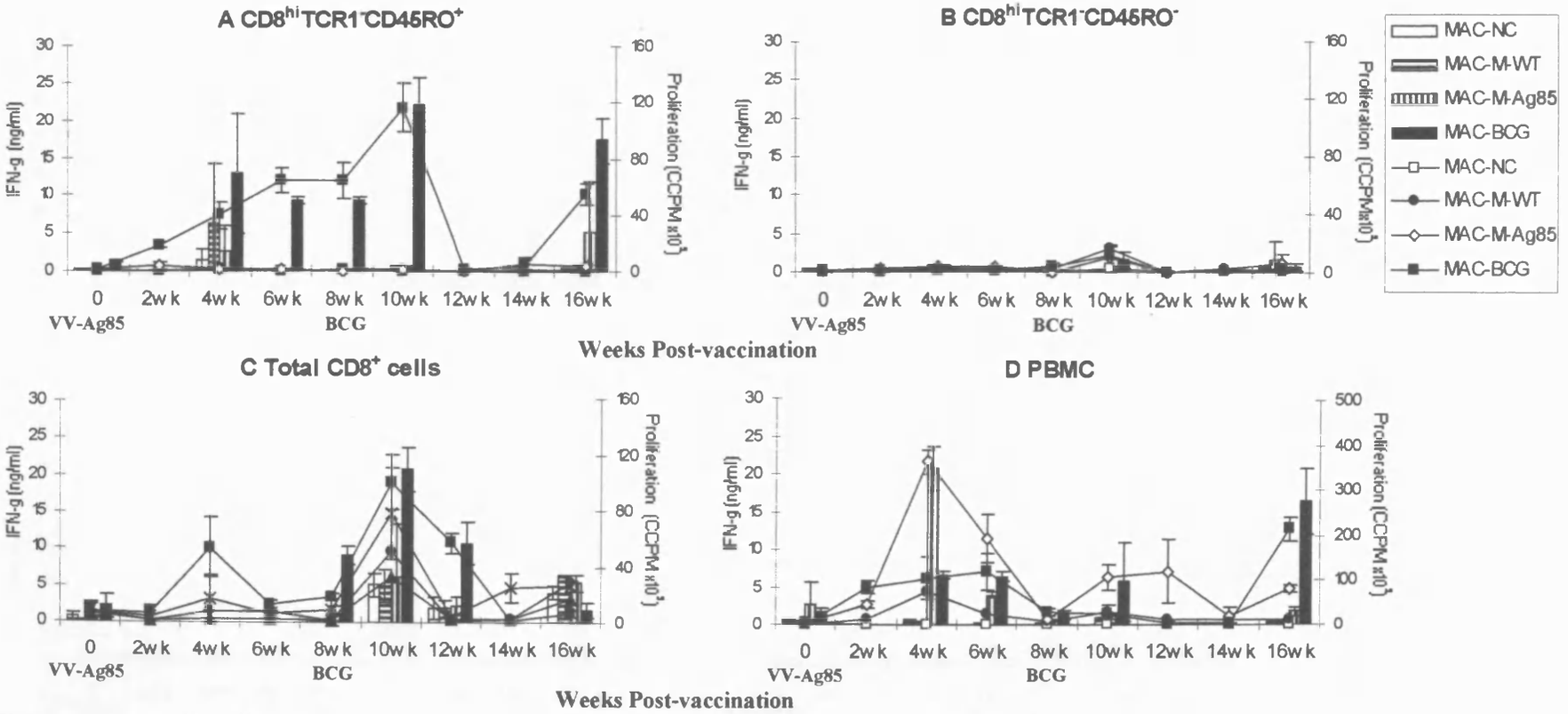


Figure 5.5.1 Proliferation and production of IFN-γ by T cells from an animal (697) vaccinated with vaccinia virus expressing Ag85 (VV-Ag85) and boosted with BCG at 8 wks post-vaccinia. $CD8^+$ cells were isolated from PBMC using MACS paramagnetic beads. $CD8^+$ cells were stained with antibodies to the γδ TCR (TCR1) and CD45RO and were sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected (MAC-NC), MVA-WT-infected (0.5 pfu), MVA-Ag85-infected (0.5 pfu) or BCG-infected Mϕ (MAC). Proliferation was measured by incorporation of ^3H TdR shown as CCPM (line) and production of IFN-γ was measured in the supernatant using ELISA shown in ng/ml (bar). The mean and standard deviation are shown for triplicate samples.

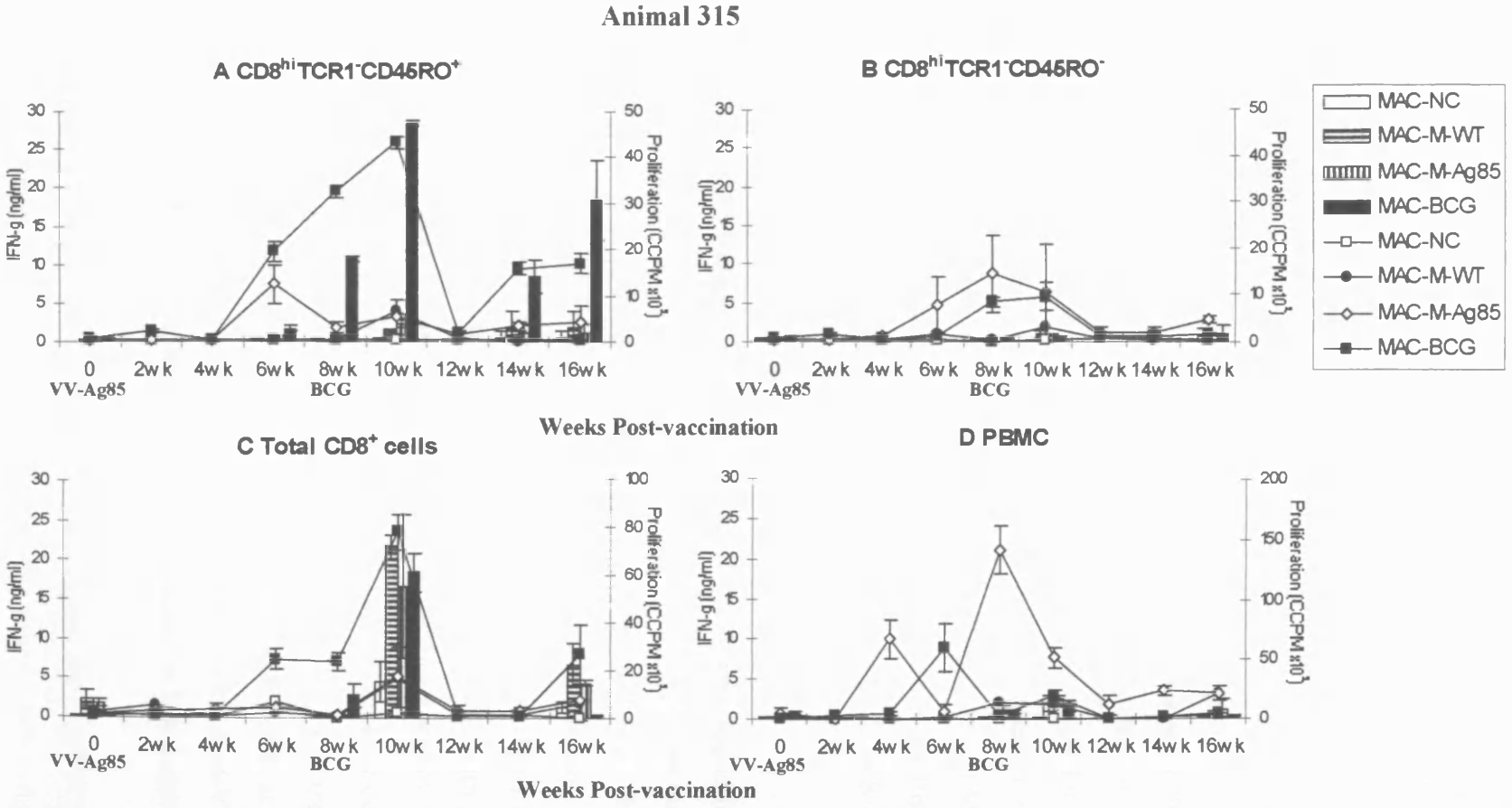


Figure 5.5.2 Proliferation and production of IFN-γ by T cells from an animal (315) vaccinated with vaccinia virus expressing Ag85 (VV-Ag85) and boosted with BCG. $CD8^+$ cells were isolated from PBMC using MACS paramagnetic beads. $CD8^+$ cells were stained with antibodies to the γδ TCR (TCR1) and CD45RO and were sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected (MAC-NC), MVA-WT-infected (0.5 pfu), MVA-Ag85-infected (0.5 pfu) or BCG-infected Mϕ (MAC). Proliferation was measured by incorporation of ^3H TdR shown as CCPM (line) and production of IFN-γ was measured in the supernatant using ELISA shown in ng/ml (bar). The mean and standard deviation are shown for triplicate samples.

Mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}T$ cells were detected again at 16 wks post-VV-Ag85 (8wks post-BCG) after culture with BCG-infected Mϕ (Fig 5.5.1A).
No response after vaccination with VV-Ag85 was observed in the $CD8^{hi}TCR1^{-}CD45RO^{-}T$ cells from animal 697. After vaccination with BCG a low level response to mycobacteria was observed at 2 wks post-BCG (10wks post-VVAg85) (Fig 5.5.1B).

A transient proliferative response was detected in the total $CD8^{+}$ cells from animal 697 at 4 wks post-vaccination with VV-Ag85A. It is shown in figure 5.5.1C that vaccination with BCG at 8 wks post-VV-Ag85, boosted the response of the total $CD8^{+}$ cells. At 2 wks post-BCG vaccination (10wks post-VV-Ag85) these cells proliferated and produced IFN-γ after culture with BCG-infected Mϕ. This response of the total $CD8^{+}$ cells declined again at 8wks post-BCG (16wks post-VV-Ag85) (Fig 5.5.1C). Vaccination with VV-Ag85 induced the PBMC from animal 697 to respond to MVA-Ag85-infected Mϕ which peaked at 4wks post-VV-Ag85. However, the response to Ag85 detected in the PBMC declined after 4 wks post-VV-Ag85 and was boosted by vaccination with BCG at 8 wks post-VV-Ag85 (Fig 5.5.1D).

The $CD8^{hi}TCR1^{-}CD45RO^{+}T$ cells from animal 315 were found to respond to vaccination with VV-Ag85 at 6 wks post-vaccination. Figure 5.5.2A shows that these cells proliferated after culture with both MVA-Ag85 and BCG infected Mϕ. The peak of the response induced by VV-Ag85 occurred at 8 wks post-vaccination. At this time-point the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells proliferated and produced IFN-γ only after re-stimulation with BCG-infected Mϕ. When the animals were inoculated with BCG at 8 wks post-VV-Ag85, this boosted the response of the $CD8^{hi}TCR1^{-}CD45RO^{+}T$ cells as shown by an increase in the level of proliferation and IFN-γ production after culture with BCG-infected Mϕ (10wks post-VV-Ag85). This boosting effect of BCG was transient as no response to mycobacteria was detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}T$ cells at 4 wks post-BCG (12wks post-VV-Ag85). However, at 6wks post-BCG (14wks post-VV-Ag85) mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells were detected in blood again (Fig 5.5.2A).

In animal 315, at 8 wks post-VV-Ag85 vaccination, the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells are shown in figure 5.5.2A to proliferate in response to MVA-Ag85-infected and BCG-

infected Mϕ. This response in the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells was not boosted by BCG and had returned to background levels at 12 wks post-VV-Ag85 (4wks post-BCG)(Fig 5.5.2B).

Vaccination with the VV-Ag85 induces a response in the total $CD8^{+}$ cells from animal 315 as shown by proliferation after culture with BCG-infected Mϕ at 6 and 8 wks post-vaccination. At 2 wks post-BCG (10wks post-VV-Ag85) the response in the $CD8^{+}$ cells had increased greatly and these cells were found to proliferate and produce IFN-γ after culture with Mϕ infected with BCG, MVA-WT and MVA-Ag85. The fact that the $CD8^{+}$ cells responded to MVA-WT suggests that this response may not be specific for a mycobacterial antigen. The boosting effect of the BCG on the $CD8^{+}$ cells was short-lived as no response was detected in these cells at 4 wks post-BCG (12 wks post-VV-Ag85) (Fig 5.5.2C).

Vaccination with VV-Ag85 induced the PBMC from animal 315 to proliferate after culture with MVA-Ag85 infected Mϕ, this response was first detected at 4 wks post-VV-Ag85 and peaked at 8 wks. By 10 wks post-VV-Ag85 (2wks post-BCG) the response in the PBMC declined and no boosting effect was observed after BCG (Fig.5.5.2D).

5.5.2 Specific lysis of BCG-infected Mϕ by $CD8^{hi}TCR1^{-}$ T cells from animals primed with VV-Ag85 and boosted with BCG.

The capacity of mycobacteria-reactive $CD8^{+}$ T cells from animals 315 and 697 to specifically lyse BCG-infected Mϕ was investigated. The $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells were isolated as described previously from blood at 11 wks post-VV-Ag85 (3 wks post-BCG). The cytotoxicity of these cells was measured using the ^{51}Cr release assay as described in the materials and methods.

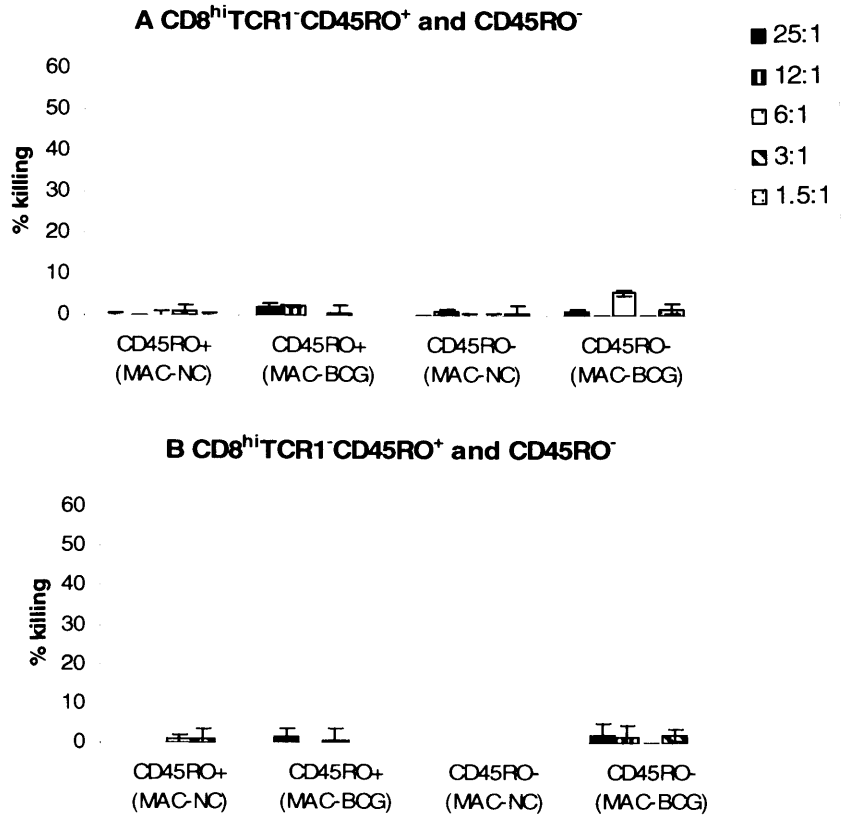


Figure 5.5.3 Killing of BCG-infected Mϕ by $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from two animals 697 (A) and 315 (B) vaccinated with VV-Ag85 and boosted with BCG. $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells were sorted from blood at 3 wks post-BCG (11 wks post-vaccinia) as described previously. Cells were stimulated with BCG-infected Mϕ for 5 days. Freshly prepared BCG infected (MAC-BCG) and uninfected Mϕ (MAC-NC) were labelled with ^{51}Cr and added to the $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells at different effector to target ratios for 4.5 hrs. The level of ^{51}Cr released by the labelled Mϕ into the supernatant was measured and the percentage killing was calculated as described in the materials and methods. The mean and standard deviation of triplicate samples are shown.

The results in figure 4.5.3 show that neither the $CD8^{hi}TCR1^{-}CD45RO^{+}$ or $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells killed BCG-infected or uninfected Mϕ in this assay. The results may reflect the fact that no proliferation or IFN-γ production could be detected in either subset after culture with BCG-infected Mϕ at 12 wks post-VV-Ag85 (4 wks post-BCG) (figure 5.5.2 and 5.5.1).

5.6 Analysis of perforin expression by $CD8^{+}CD3^{+}$ T cell in the draining LN after inoculation of BCG in BCG-vaccinated animals

To investigate whether the mycobacteria-reactive $CD8^{hi}TCR1^{-}$ T cells induced by BCG-vaccination mediate memory responses to mycobacteria *in vivo,* the following model was used in which at 8 wks post-BCG vaccination animals were challenged intradermally either with BCG or PBS. Three days later the draining lymph node (prescapular) was removed and analysed for expression of CD8, CD3 and perforin using 3-colour immunofluorescence and confocal microscopy.

The results show that $CD8^{+}CD3^{+}Perforin^{+}$ T cells could be readily detected in the sections of LN from BCG-vaccinated animals that had been inoculated with BCG (Fig 5.5.1-3), whilst this was not the case in LN isolated from the BCG-vaccinated inoculated intradermally with PBS (Fig 5.5.4-6). In the LN containing $CD8^{+}CD3^{+}Perforin^{+}$ T cells, these cells appear to be clustered together in specific areas of the LN and the perforin appears to be concentrated in granules within cells rather than being dispersed throughout the cell. It was also noted that almost all of the $perforin^{+}$ cells in the LN were $CD8^{+}CD3^{+}$. No staining was observed with the isotype controls in any of the sections used in this study (Fig 5.5.7-8)

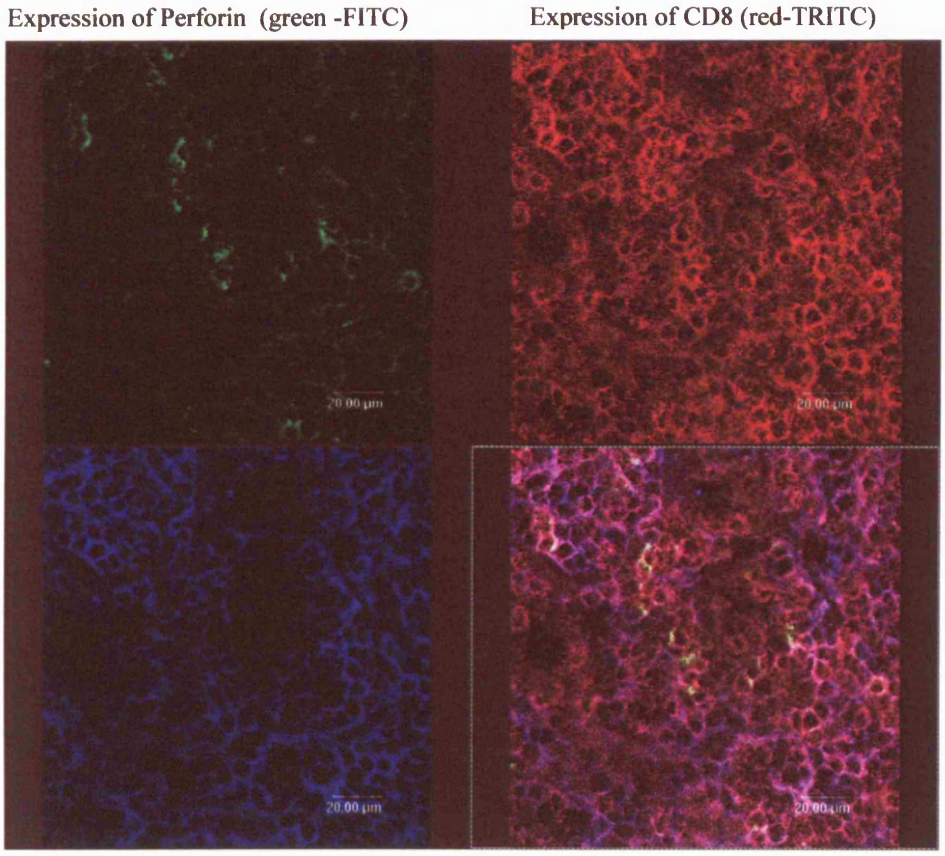


Figure 5.6.1. Immunofluorescence staining of the draining LN from a BCG-vaccinated animal (904) after intradermal challenge with BCG. The animal was vaccinated with 10^6 BCG, at 8 wks post-vaccination the animal was challenged with 10^6 BCG intradermally. The prescapular LN was removed 3 days after challenge and cut into 1 cm^3 squares before being immediately cryopreserved. The sections were cut onto slides and stained with antibodies to CD8 (red-TRITC), CD3 (blue-Alexa fluor-633) and perforin (green-FITC). The slides were then analysed on a confocal microscope. The picture shown is representative of what was viewed under the microscope.

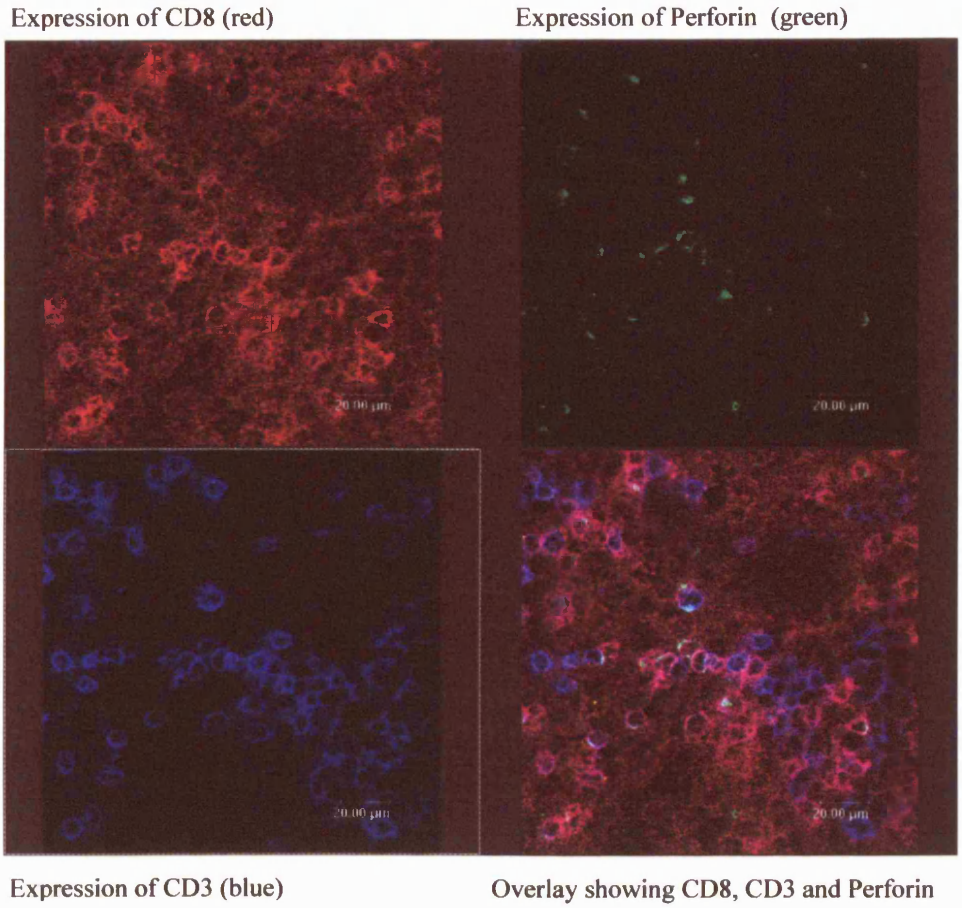


Figure 5.6.2. Immunofluorescence staining of the draining LN from a BCG-vaccinated animal (074) after intradermal challenge with BCG. The animal was vaccinated with 10^6 BCG, at 8 wks post-vaccination the animal was challenged with 10^6 BCG intradermally. The prescapular lymph nodes were removed 3 days after challenge and cut into 1 cm^3 squares before being immediately cryopreserved. The sections were cut onto slides and stained with antibodies to CD8 (red), CD3 (blue) and perforin (green). The slides were then analysed on a confocal microscope. The picture shown is representative of what was viewed under the microscope.

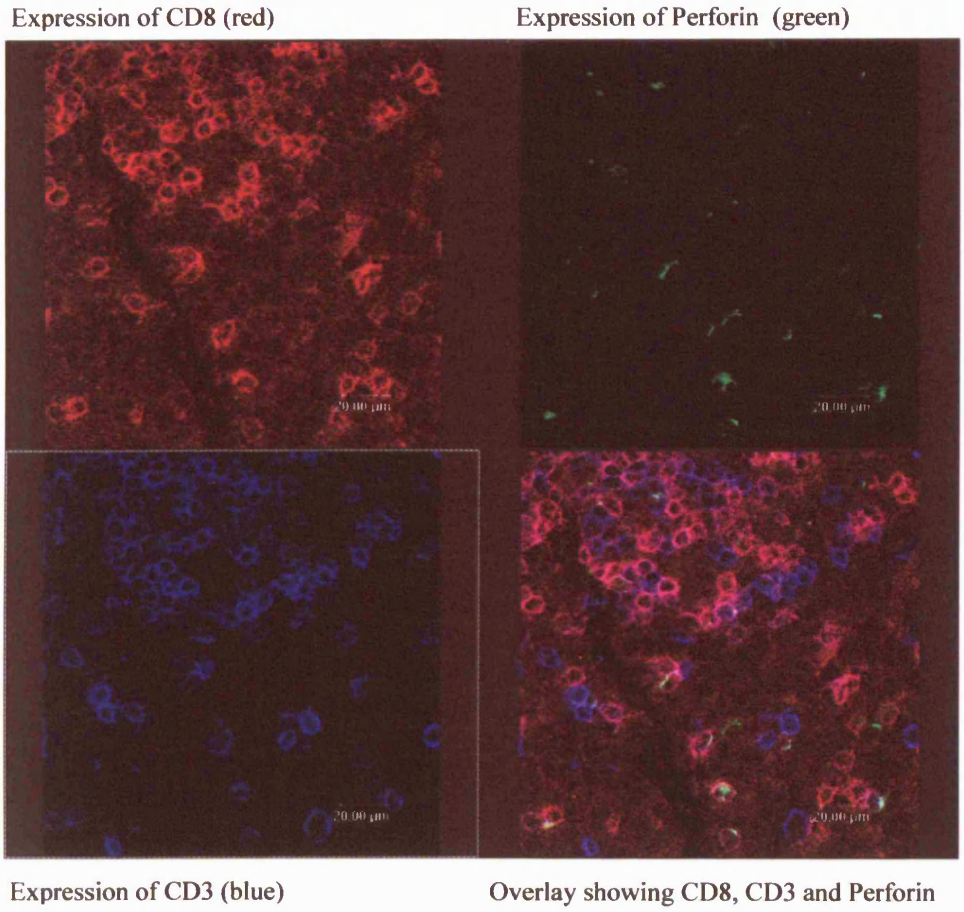


Figure 5.6.3. Immunofluorescence staining of the draining LN from a BCG-vaccinated animal (070) after intradermal challenge with BCG. The animal was vaccinated with 10^6 BCG intramuscularly, at 8 wks post-vaccination the animal was challenged with 10^6 BCG intradermally. The prescapular lymph nodes were removed 3 days after challenge and cut into 1 cm^3 squares before being immediately cryopreserved. The sections were cut onto slides and stained with antibodies to CD8 (red), CD3 (blue) and perforin (green) The slides were then analysed on a confocal microscope. The picture shown is representative of what was viewed under the microscope.

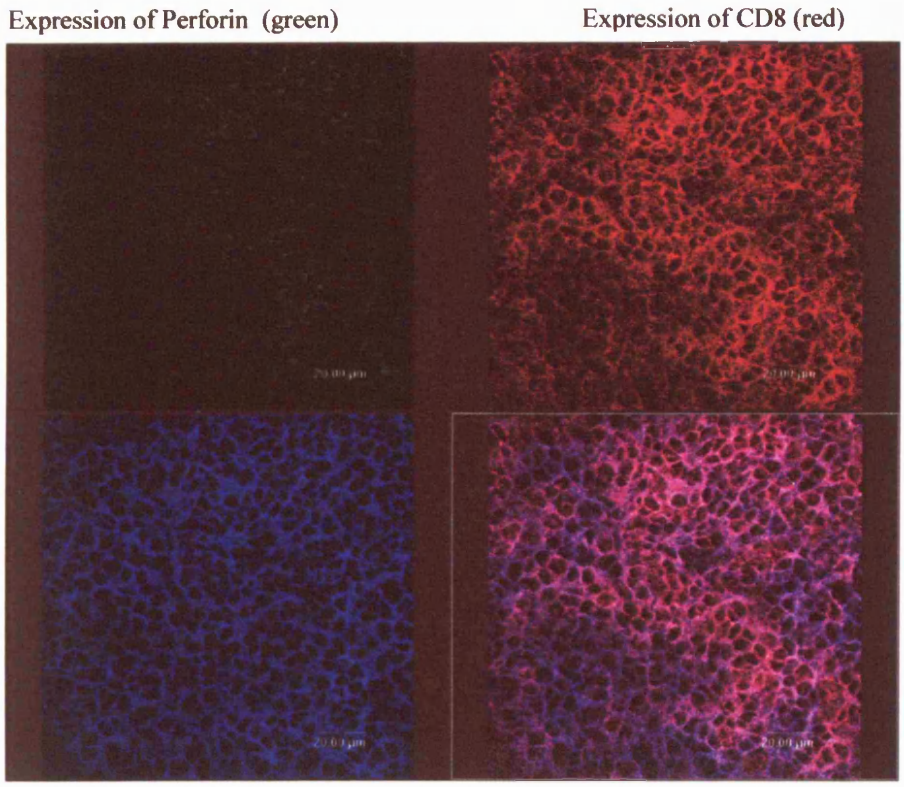


Figure 5.6.4. Immunofluorescence staining of the draining LN from a BCG-vaccinated animal (907) after intradermal challenge with PBS. The animal was vaccinated with 10^6 BCG IM, at 8 wks post-vaccination the animal was challenged with 100 µl of PBS intradermally. The prescapular lymph nodes were removed 3 days after challenge and were cut into 1 cm^3 squares before being immediately cryopreserved. The sections were cut onto slides and stained with antibodies to CD8 (red), CD3 (blue) and perforin (green). The slides were then analysed on a confocal microscope. The picture shown is representative of what was viewed under the microscope.

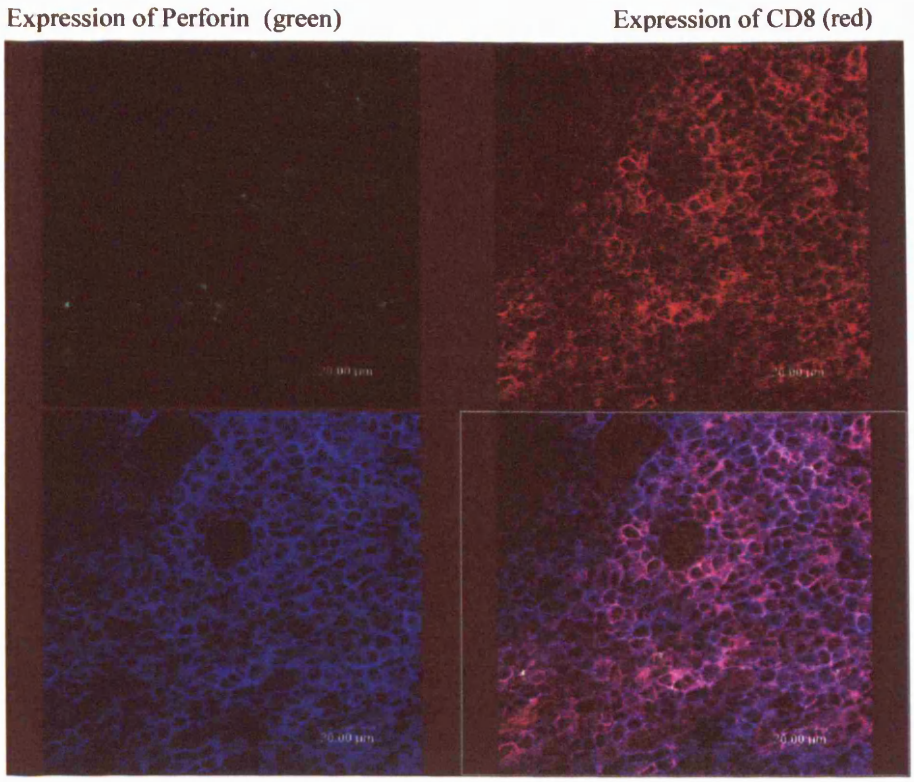


Figure 5.6.5. Immunofluorescence staining of the draining LN from a BCG-vaccinated animal (923) after intradermal challenge with PBS. The animal was vaccinated with 10^6 BCG IM, at 8 wks post-vaccination the animal was challenged with 100 µl of PBS intradermally. The prescapular lymph nodes were removed 3 days after challenge and were cut into 1 cm^3 squares before being immediately cryopreserved. The sections were cut onto slides and stained with antibodies to CD8 (red), CD3 (blue) and perforin (green). The slides were then analysed on a confocal microscope. The picture shown is representative of what was viewed under the microscope.

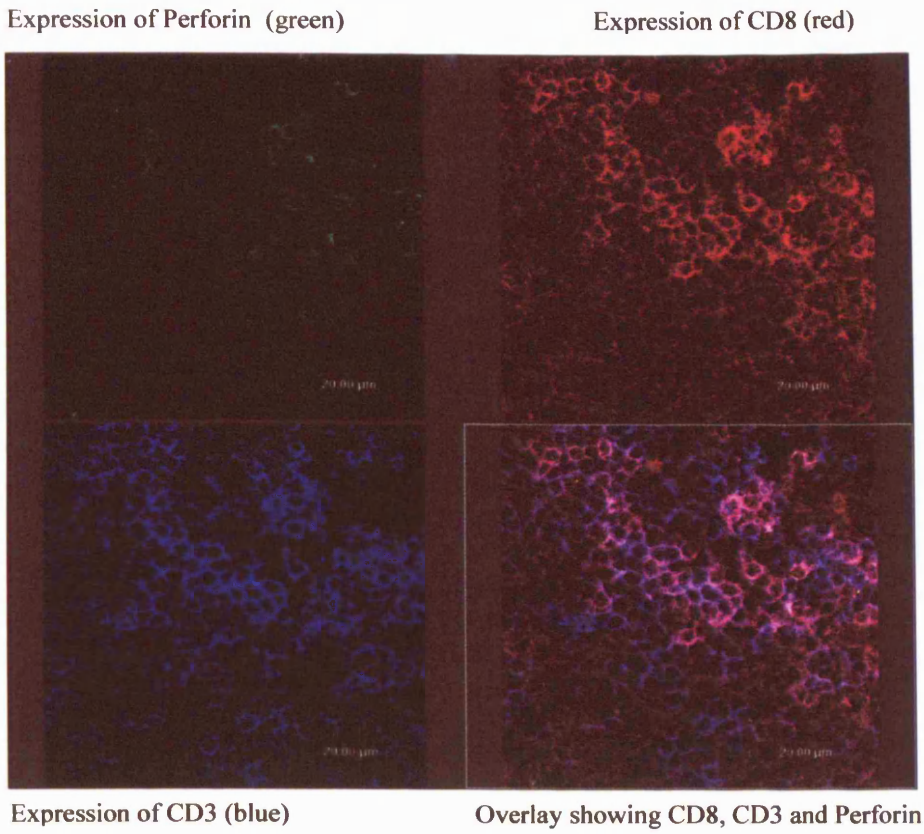


Figure 5.6.6. Immunofluorescence staining of the draining LN from a BCG-vaccinated animal (932) after intradermal challenge with PBS. The animal was vaccinated with 10^6 BCG IM, at 8 wks post-vaccination the animal was challenged with 100 µl of PBS intradermally. The prescapular lymph nodes were removed 3 days after challenge and were cut into 1 cm^3 squares before being immediately cryopreserved. The sections were cut onto slides and stained with antibodies to CD8 (red), CD3 (blue) and perforin (green). The slides were then analysed on a confocal microscope. The picture shown is representative of what was viewed under the microscope.

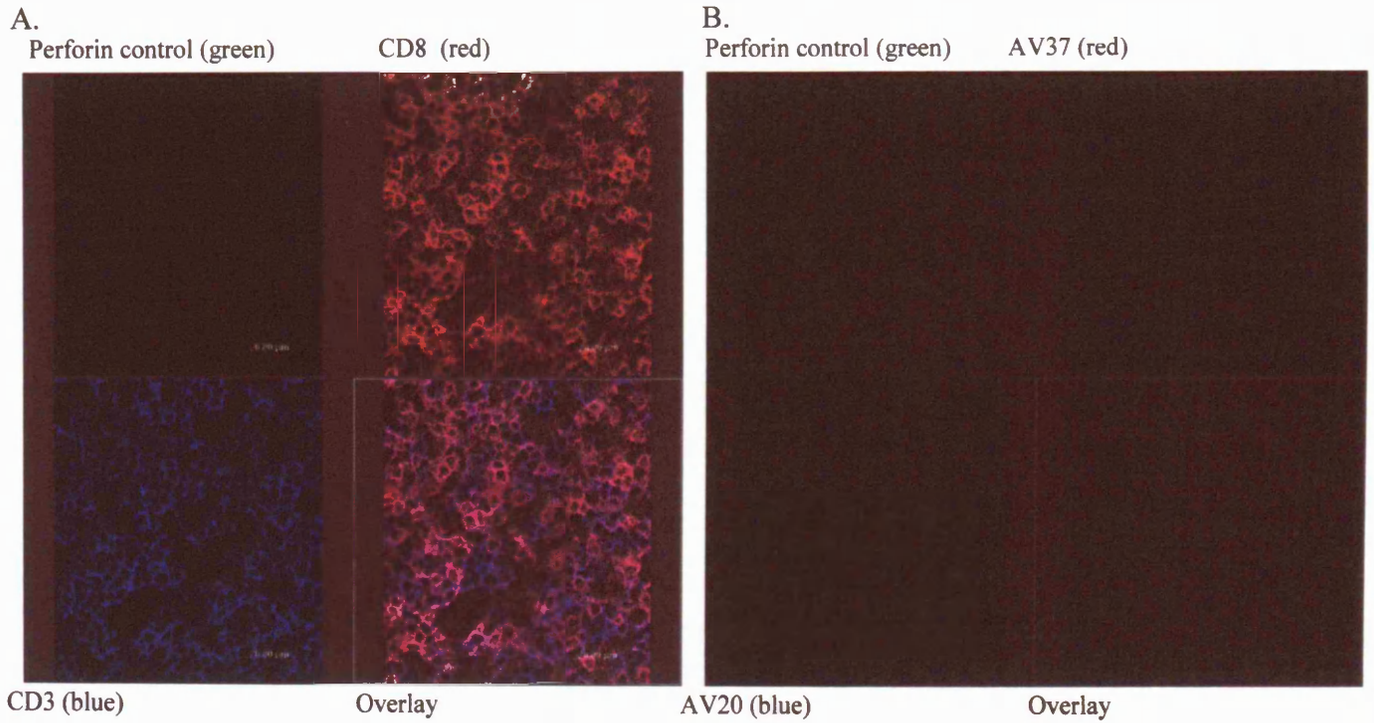


Figure 5.6.7. Immunofluorescence staining of the draining LN from a BCG-vaccinated animal (904) after intradermal challenge with BCG. The animal was vaccinated with 10^6 BCG IM, at 8 wks post-vaccination the animal was challenged with 10^6 BCG intraderrmally. The prescapular lymph nodes were removed 3 days after challenge and were cut into 1 cm^3 squares before being immediately cryopreserved. The sections were cut onto slides and stained with antibodies to CD8 (red), CD3 (blue) and perforin control (green) (A) or with isotype controls AV29 (red), AV20 (blue) and perforin control (green) (B) supplied from BD Pharmingen. The slides were then analysed on a confocal microscope. The picture shown is representative of what was viewed under the microscope.

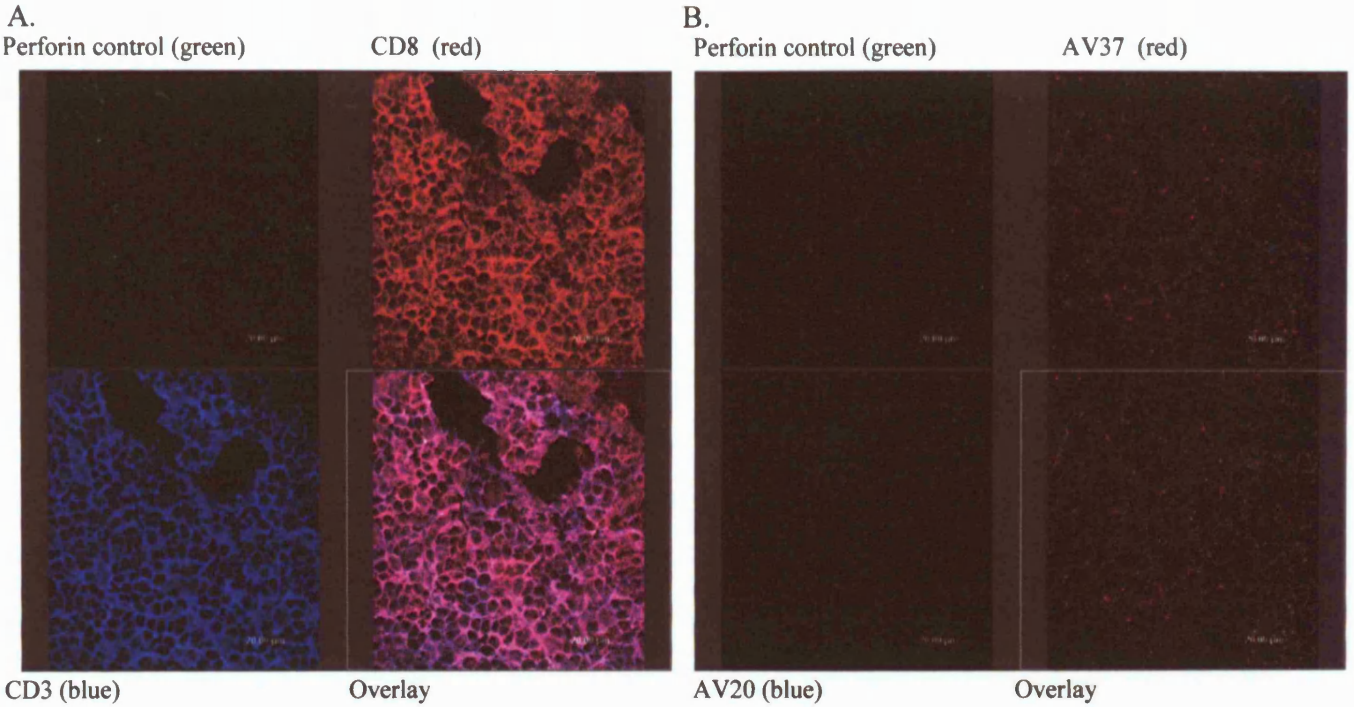


Figure 5.6.8. Immunofluorescence staining of the draining LN from a BCG-vaccinated animal (932) after intradermal challenge with PBS. The animal was vaccinated with 10^6 BCG IM, at 8 wks post-vaccination the animal was challenged with 100 µl of PBS intradermally. The prescapular lymph nodes were removed 3 days after challenge and were cut into 1 cm^3 squares before being immediately cryopreserved. The sections were cut onto slides and stained with antibodies to CD8 (red), CD3 (blue) and perforin control (green) (A) or with isotype controls AV29 (red), AV20 (blue) and perforin control (green) (B) supplied from BD Pharmingen. The slides were then analysed on a confocal microscope. The picture shown is representative of what was viewed under the microscope.

5.7 Discussion

It is known that BCG vaccination elicits mycobacteria-reactive T cells that in many trials have afforded a high level of protection against infection with *M. bovis*. The methods used to detect vaccine-induced responses in cattle, most commonly involve the culture of either whole blood or PBMC with PPD-B. In past studies, the response detected in the PBMC, peaked at between 2-4 wks post-vaccination. The use of PPD-B to stimulate the PBMC and the time frame of the response detected suggests that the methods used are mostly measuring $CD4^+$ T cell responses.

One study has reported that $CD8^+$ cells from BCG-vaccinated animals responded more highly to BCG-infected or PPD-B-pulsed DC compared to non-vaccinated animals (Hope, Sopp et al. 2002). It was shown in Chapter 4 that the $CD8^+$ cell population in cattle is comprised of NK cells, αβ T cells and γδ T cells. In the study by Hope et al the responding cell type within the total $CD8^+$ population was not defined (Hope, Sopp et al. 2002). Therefore this study may have been measuring a combination of responses from the three cell-types, illustrating that the analysis of $CD8^+$αβ T cell response will require cell-sorting. The aim of this chapter was to investigate the development of mycobacteria-reactive $CD8^+$αβ T cell responses in cattle after vaccination with BCG.

In this chapter, the $CD8^+$ population of cells was dissected and $CD8^{hi}TCR1^-CD3^+$ T cells, most likely to be the αβ T cells were isolated from blood before and after BCG vaccination. Mycobacteria-reactive $CD8^+$ T cells were detected after culture with BCG-infected APC by measuring the level of proliferation and production of IFN-γ. The $CD8^+$ T cells and APC were cultured for 5 days as this culture period has been shown to result in the activation and proliferation of mycobacteria-specific T cells. (Carpenter, Fray et al. 1997; Hope, Kwong et al. 2000). Analysis of immune responses after 5 days in culture is likely to measure the response of central memory T cells. It is thought that the BCG-specific effector memory cells present in the sample are likely to have undergone apoptosis by the end of the 5 day culture period as effector memory T cells have been proposed to respond immediately upon recognition of antigen. In contrast, central memory T cells have been proposed to have limited effector functions. Upon recognition of antigen central memory T cells proliferate giving rise to effector T cells that have the capacity to exert effector functions. Therefore, the proliferation measured in these experiments is likely to have resulted from the central memory T cells and the

IFN-γ present in the supernatants will have been produced by both the effector memory T cells present in the sample and the effector T cells generated by the central memory T cells. The direct analysis of mycobacteria-reactive effector memory T cells would require a much shorter culture period and the effector functions of the cells would have to be measured *ex vivo*. At present, the tools used in human and mouse studies to measure effector and effector memory T cells responses such as MHC tetramers or immunodominant peptides are presently not available for studies in cattle.

After vaccination with BCG, mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells were detected in blood from 3 mth old calves. The cells proliferated after culture with BCG-infected Mϕ but not PPD-B pulsed Mϕ. This is similar to observations in BCG-vaccinated humans in which the BCG-specific $CD8^+$ T cells responded more highly to BCG than PPD-B (Lewinsohn, Alderson et al. 1998; Smith, Malin et al. 1999; Tsunetsugu-Yokota, Tamura et al. 2002). The magnitude of the response to mycobacteria detected in the $CD8^{hi}TCR1^-$ T cells peaked at 8 wks post-vaccination whereas the response of PBMC peaked at 2 wks post-vaccination. The differences observed in the kinetics of the responses suggest that the main population of responding cells in the PBMC are not $CD8^{hi}TCR1^-$ T cells but possibly $CD4^+$ T cells as they also respond to PPD-B.
The pattern of response to mycobacteria detected in the total $CD8^+$ population did not reflect that observed in the $CD8^{hi}TCR1^-$ T cells. The total $CD8^+$ cells responded strongly to PPD-B after vaccination with BCG. It is possible that the responding cells are γδ T cells as mycobacterial-reactive γδ T cells have been shown previously to proliferate in response to PPD-B (Rhodes, Hewinson et al. 2001). This demonstrates that the total $CD8^+$ population cannot be used to accurately measure $CD8^+$ αβ T cell responses in cattle.

The mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells induced by BCG vaccination were further characterised in three animals that had been vaccinated at 6 mth of age. In the three animals, BCG-infected Mϕ were more effective at stimulating immune responses *in vitro* compared to BCG-infected DC. It is likely that the monocyte-derived Mϕ are more phagocytic than monocyte-derived DCs. This increased rate of phagocytosis in the Mϕ may result in an enhanced rate of presentation of BCG antigens on MHC class I molecules. It has also been shown that the membrane permeability of phagosomes in

BCG-infected Mϕ is increased, thus aiding the release of mycobacterial antigens into the cytosol and access to the MHC class I processing and presentation pathway (Teitelbaum, Cammer et al. 1999).

In all three animals, after vaccination with BCG, mycobacteria-reactive $CD8^{hi}TCR1^{-}$ $CD45RO^{+}$ T cells were detected in blood. These cells proliferated and produced IFN-γ after culture with BCG-infected Mϕ and to a lesser extent BCG-infected DCs. The kinetics and magnitude of this response varied slightly between the animals but was found to peak at between 7-12 wks post-vaccination.

No response was detected in the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells, this is in agreement with the results of chapter 3, indicating that the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells in blood resemble naïve T cells, in terms of phenotype and lack of expression of effector molecules. In addition, RT-PCR analysis showed that CCR7 is predominantly expressed by $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells and that these cells lack expression of perforin and Bolysin. Expression of CCR7 is required for circulation through the LN and is mainly expressed by naïve T cells with the exception of a small population of memory cells that reside in the LN (Wills, Okecha et al. 2002; Bromley, Thomas et al. 2005). These results demonstrate that the population of $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells in blood contains a high proportion of naïve T cells.

RT-PCR analysis showed that perforin was expressed by mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animals 614, 645 and 249, indicating that these cells have the capacity to be cytotoxic. The mRNA band for perforin was stronger in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells cultured with BCG-infected Mϕ compared to uninfected Mϕ, suggesting that an antigen-dependent up-regulation of perforin expression by the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells.

Bolysin was found to be only expressed by mycobacteria-reactive $CD8^{hi}TCR1^{-}$ $CD45RO^{+}$ T cells from animals 614 and not by those cells from animals 645 and 249. Furthermore, expression of Bolysin was only observed in $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells after culture with BCG-infected Mϕ suggesting that expression of this molecule is more tightly regulated than perforin as $CD8^{hi}TCR1^{-}CD45RO^{+}$ $Perforin^{+}$ T cells are observed *ex vivo* without any stimulation. These results suggest that the mycobacteria-

reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animal 614 induced by BCG-vaccination are likely to have a greater ability to kill mycobacteria compared to the same cells from animals 645 and 249.

To protect the cytotoxic cells that express bolysin from its harmful effects, bolysin is present in two forms an active 9 KDa molecule and inactive 15 KDa molecule. The lack of expression of Bolysin in animals 645 and 249 may be attributed to the kinetics of its expression and these cells may be expressing the inactive 15kDa Bolysin. It is also possible that the mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animals 645 and 249 have not acquired the ability to express granulysin as expression of perforin and granulysin appears to be differentially regulated. Stenger and co-workers reported that perforin was required to facilitate access of granulysin inside the infected cells to kill the mycobacteria (Stenger, Hanson et al. 1998). Whilst, Canaday and co-workers reported that inhibition of perforin and FASL did not affect the ability of human *M. tuberculosis*-specific $CD8^{+}$ T cells to reduce mycobacteria growth within Mϕ (Canaday, Wilkinson et al. 2001). This suggests that either granulysin can enter infected cells in the absence of perforin or that *M. tuberculosis* specific T cells can inhibit mycobacterial growth through a number of mechanisms, some of which are independent of perforin and FASL (Lammas, Stober et al. 1997; Russell and Ley 2002).

The RT-PCR analysis indicated that perforin expression was up-regulated in the BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells. This was confirmed using flow cytometry, which demonstrated that an increased percentage of $CD8^{hi}TCR1^{-}$ T cells express perforin after culture with BCG-infected Mϕ compared to control Mϕ. The $CD8^{hi}TCR1^{-}$ T cells induced to express perforin also expressed CD3 and CD45RO. Most of these cells had lost expression of CD62L and CD28 with exception of a small percentage of $CD8^{hi}TCR1^{-}perforin^{+}$ T cells from animals 249 and 645. It is possible that these cells are not fully differentiated or that these cells may be central memory T cells. This is similar to studies in BCG-vaccinated humans that showed $CD8^{+}CD45RA^{-}$ $Perforin^{+}$ T cell expanded after culture with BCG-infected DCs (Tsunetsugu-Yokota, Tamura et al. 2002).

Interestingly, a higher percentage of BCG-reactive $CD8^{hi}TCR1^{-}$ T cells from animal 249 up-regulated perforin expression compared to animals 614 and 645. Whilst the BCG-reactive $CD8^{hi}TCR1^{-}$ T cells from 614 and 645 proliferated to a greater extent

and produced more IFN-γ than in animal 249. The results suggest a disassociation of perforin expression and the ability to produce IFN-γ and proliferate in response to antigen. In humans inhibition of IFN-γ did not have any effect on the expansion of perforin expressing $CD8^+$ T cells. Furthermore, in IFN-γ KO mice, Ag-specific $CD8^+$ T cells express perforin (Nguyen, van Ginkel et al. 2000; Tsunetsugu-Yokota, Tamura et al. 2002). It is should also be noted that a higher percentage of $CD8^{hi}TCR1^-$T cells from animal 249 expressed perforin prior to culture with Mϕ compared to animals 614 and 645 this may, in part, account for the differences observed.

BCG-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells from animals 614 and 645 were found to specifically lyse BCG-infected Mϕ. Surprisingly, no cytolytic activity was detected in the $CD8^{hi}TCR1^-CD45RO^+$ T cells from animal 249 even though these cells responded to vaccination and up-regulated perforin expression after culture with BCG-infected Mϕ.

It is possible that the specific killing measured by the ^{51}Cr assay is not induced by exocytosis of cytotoxic granules and the BCG-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells may be killing the infected Mϕ via expression of cell membrane molecules such as FASL. In addition, the $CD8^{hi}TCR1^-CD45RO^+$ cells from the three animals were cultured with Mϕ for 5 days and it is likely that the optimal length of time for stimulation of T cells to exhibit killing may differ between animals. It would be interesting to investigate the ability of the BCG-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells to exhibit killing *ex vivo*, as it is possible that the BCG-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells from animal 249 that express high levels of perforin are effector memory T cells. Whereas the BCG-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cell populations from animals 614 and 645 may contain more central memory T cells. The fact that central memory T cells have a greater capacity to proliferate and produce effector T cells, this subset has been proposed to be more effective at mediating protective immunity compared to effector memory T cells (Wherry, Teichgraber et al. 2003). It is likely that the subset of memory T cells elicited by vaccination determines the effectiveness of the vaccine-induced response at providing protection against infection. This hypothesis could have been tested if these three BCG-vaccinated animals 249, 614 and 645 had been infected with *M. bovis* and the effectiveness of the vaccine-induced immune responses could have been compared.

Sensitisation to mycobacteria from exposure to some strains of environmental mycobacteria has been proposed to adversely affect the protective nature of BCG against TB (Brandt, Feino Cunha et al. 2002; de Lisle, Wards et al. 2005). Therefore vaccination of neonates is a way of minimising the risk of vaccine failure due to pre-sensitisation to environmental mycobacteria. In this study, a heightened immune response to mycobacteria was detected in the neonates prior to vaccination. The T cells from these animals responded equally to both *M. avium*-infected and BCG-infected Mϕ. This suggests that the 1 mth old animals used in these experiments had been exposed to *M. avium* prior to vaccination. This reactivity to mycobacteria in very young unvaccinated animals, has been reported previously and could be detected in nonvaccinated animals up until 9 wks of age (Olsen and Storset 2001; Buddle, Wedlock et al. 2003; Skinner, Wedlock et al. 2005). It should also be noted that no response was detected to mycobacteria prior to vaccination of 6 mth old animals. It is possible that the immune cells from very young animals are hyper-responsive to pathogens in order to compensate for the lack of memory immune cells. Therefore the reactivity to mycobacteria observed in young non-vaccinated animals may be due to a hyperresponsive immune response to the mycobacteria in either the Mϕ or T cells.

The immune response detected prior to vaccination in animals 23 and 29 declined sharply after vaccination with BCG. However, at 5-7 wks post-vaccination the response to mycobacteria increased in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from these animals suggesting the development of a vaccine-induced response. The BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cell response peaked at between 7-9 wks post-vaccination in the three animals 23, 29 and 34. This is comparable to the animals that were vaccinated with BCG at 6 mths, in which the response of the BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cell peaked at between 7-12 wks post-vaccination. At the peak of the response, the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from the neonates responded slightly higher to BCG than to *M. avium*-infected Mϕ. As the response to both BCG and *M. avium* was comparable prior to the peak of the response, vaccination appeared to bias the response slightly towards BCG antigens. This is consistent with previous reports that showed prior sensitisation of cattle to *M. avium* did not inhibit the immune response induced by BCG vaccination but did bias it towards common antigens between the two mycobacteria (Howard, Kwong et al. 2002). Furthermore, animals vaccinated at 6 wks

of age that are responsive to *M. avium* are equally protected against disease as animal vaccinated at birth (Buddle, Wedlock et al. 2003).

The BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells induced by vaccination in the three neonates, proliferated and produced IFN-γ in response to live mycobacteria. However, only BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animals 29 and 34 specifically lysed BCG-infected Mϕ and no CTL activity was detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animal 23. These results suggest that the mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cell responses from animals 29 and 34 may be more protective than those from animal 23 as lysis of infected Mϕ has been shown to result in a reduction in mycobacterial numbers (Oddo, Renno et al. 1998).

A low level transient response was detected post-vaccination in the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells from the three neonates. This finding was unexpected as no response was detected in these cells from the older vaccinated animals. It is possible that the responding cells are a contaminating population within the sorted $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells. However, the purity of sorted populations was typically greater than 98% and the sorted $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells were found to be 100% $CD3^{+}$. Another possibility is that the observed response is due to a population of $CD45RA^{+}$ primed cells within the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells. These cells have been shown to arise during chronic infections (Wills, Carmichael et al. 1999). It is known that *M. avium* establishes a chronic infection in cattle which is typically asymptomatic (Tessema, Koets et al. 2001). The strong responses observed to *M. avium* in the neonatal animals suggests that these calves may have been infected with *M. avium*.

Heterologous prime-boost strategies involving priming with BCG and boosting with recombinant DNA, protein or viral vectors expressing mycobacterial antigens have been shown to induce a more robust immune response involving both memory $CD4^{+}$ and $CD8^{+}$ T cells compared to a single dose of BCG. Vaccination with virulent vaccinia can provide life-long immunity with memory T cells being present some 30 yrs post-vaccination (Demkowicz, Littaua et al. 1996). Vaccinia virus is a prime vehicle for the delivery of mycobacterial antigens as it replicates within the cytoplasm of infected cells, allowing access to the MHC class I processing machinery. Two animals were vaccinated with VV expressing Ag85A and at 8 wks post-vaccination the animals were given BCG as previous vaccination experiments have shown that the

peak of the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cell responses occured between 7-12 wks post-vaccination. In both animals the VV-Ag85A vaccination induced $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells that responded to BCG-infected Mϕ and this response appeared to peak at 8 wks post-VV-Ag85A, just prior to BCG. Suprisingly these $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells did not respond to culture with Mϕ infected with MVA-Ag85A. However, the PBMC consistently responded to the MVA-Ag85A and due to the early kinetics of this response it is thought that the main responding population in the PBMC are $CD4^{+}$ T cells. The results suggest that the infection of Mϕ with the MVAAg85A may in this assay lead to the preferential presentation of antigens on MHC class II molecules and not MHC class I molecules. This may occur if there was a problem with the MVAAg85A as it may not be actively infecting the Mϕ and efficiently expressing the Ag85A.

Vaccination with BCG when administered at 8 wks post-VV-Ag85A, significantly boosted the response to mycobacteria induced by the VV-Ag85 in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from both animals. A ^{51}Cr release assay carried out at 3 wks post-BCG showed that no CTL activity could be detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from either animal. This lack of CTL activity may be explained by fact that the boosting effect of the BCG was short-lived and no proliferation or IFN-γ production was detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells 4 wks post-BCG. It is possible that the responding $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells migrated into tissues or may have been exhausted and undergone AICD after the BCG boost. The lack of response detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells does not appear to be due to a problem with the assay as the total $CD8^{+}$ cells and PBMC are responding at these time-points.

In animal 397, mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells were detected again at 6 wks post-BCG, whereas in animal 697these cells were not detected until 8 wks post-BCG. It is possible that the mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells induced by the VVAg85 vaccine responded immediately after BCG was administered and may have cleared most of the BCG. The second wave of responses detected at 6-8 wks post-BCG in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells may be the result of a primary $CD8^{+}$ T cell response induced by the remaining BCG.

After vaccination with BCG, responses to mycobacteria were consistently detected in the PBMC prior to being detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells. This suggests

that development of the $CD8^+$ T cell responses may require the presence of mycobacteria-reactive $CD4^+$ T cells. The responding $CD4^+$ T cells may aid the cross-presentation of BCG on MHC class I molecules *in vivo* via a number of mechanisms. The first is the activation of BCG-infected Mϕ resulting in the increased killing of the BCG and access of mycobacterial antigens into the cytosol. Second, the mycobacteria-reactive $CD4^+$ T cells may induce apoptosis of the BCG infected-Mϕ resulting in the uptake of apoptotic bodies by uninfected Mϕ. Another mechanism is the conditioning of DCs via interactions with CD40-CD40L. Previously the development of competent memory $CD8^+$ T cell responses in *M. tuberculosis* infection of mice was shown to be aided by $CD4^+$ T cells (Schoenberger, Toes et al. 1998; Wang, Santosuosso et al. 2004). The delay in the development of $CD8^+$ T cell responses may also reflect the slow-growing nature of BCG as mycobacterial antigens may access the cytosol when metabolic byproducts are released by the replicating BCG.

So far the results of this study have shown that mycobacteria-reactive $CD8^+$ T cells respond to BCG-infected Mϕ after *in vitro* culture. However, these results do not provide any evidence that these cells can respond to mycobacteria *in vivo*. To investigate this, BCG-vaccinated animals were intradermally given BCG or PBS at 8 wks post-vaccination. Three days later the draining LN was removed. It was found that the LN from the vaccinated animals that received BCG contained clusters of $CD8^+CD3^+$ $Perforin^+$ cells. These cells were not present in the draining LNs from the vaccinated animals that were given PBS. This suggests that an activation of $CD8^+CD3^+$ T cells has occurred in the draining LN after the inoculation of BCG, the rapidity of this response suggests that it is a memory T cell response. In previous experiments $CD8^+TCR1^+$ T cells in the LN did not express perforin. Therefore, it is likely that the $CD8^+CD3^+Perforin^+$ T cells are αβ T cells. To confirm these findings, a further experiment is needed in which non-vaccinated animals are challenged intradermally with BCG.

In summary, mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells were detected in blood at between 7-12 wks after BCG-vaccination of animals vaccinated at either 1 mth or 6 mths old. In response to culture with BCG-infected Mϕ, these cells proliferated,

produced IFN-γ, up-regulated expression of cytotoxic molecules and lysed BCG-infected Mϕ.

The BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells may contribute to the control of mycobacterial infection by a number of effector functions which include the production of IFN-γ which may lead to the activation of anti-mycobacterial effector functions of infected Mϕ. In addition, BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from four out of the six BCG-vaccinated animals, lysed BCG-infected Mϕ which may also lead to the increased killing of mycobacteria. The differences observed in the effector functions and magnitude of responses of the mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from the vaccinated animals used in this study may, in part, explain the variable efficacy of BCG at protecting against infection with *M. bovis*.

CHAPTER SIX: RESULTS

Development of $CD8^+$ T cell responses induced by infection with *Mycobacterium bovis*

6.1 Introduction

Control of *M. tuberculosis* and *M. bovis* infection is cell-mediated, dependent upon the interaction of $CD4^+$, $CD8^+$, γδ T cells and NK cells with infected Mϕ. The individual contribution of each T cell is thought to involve a combination of cytokine production, cytotoxic and anti-microbial activity and regulatory functions. The vital role of $CD4^+$ T cells in immunity to TB has been defined in murine models and specifically in humans, by the increased susceptibility to infection in HIV patients (Orme and Collins 1983; Orme and Collins 1984; Muller, Cobbold et al. 1987; Leveton, Barnass et al. 1989; Barnes, Bloch et al. 1991; Hopewell 1992). In mice, experiments using KO mice, adoptive transfer and depletion studies have demonstrated that $CD8^+$T cells are a vital part of the protective immune response against TB (Muller, Cobbold et al. 1987; Orme 1987; Flynn, Goldstein et al. 1992). In humans, *M. tuberculosis* -specific $CD8^+$ T cells from healthy infected patients secrete cytokines TNF-α and IFN-γ, are mycobactericidal and lyse *M. tuberculosis*-infected Mϕ (Esin, Batoni et al. 1996; Turner and Dockrell 1996; Tan, Canaday et al. 1997; Lalvani, Brookes et al. 1998; Smith, Malin et al. 1999) . In contrast, in patients with active disease the *M. tuberculosis* -specific $CD8^+$ T cells exhibit an increased production of IL-4 and decreased levels of IFN-γ and cytotoxicity, indicating that $CD8^+$ T cells are important in the control of human TB (Smith, Klein et al. 2000; Shams, Wizel et al. 2001).

The knowledge of $CD8^+$ T cell responses in bovine TB are far behind what is known in human TB, the following is a summary of the available literature describing $CD8^+$ cells in bovine TB. $CD8^+$ cells isolated from *M. bovis*-infected animals were found to proliferate and produce IFN-γ in response to stimulation with PPD-B, MBSE and *M. bovis* (Liébana, Girvin et al. 1999). Production of IFN-γ by $CD8^+$ T cells in PPD-B stimulated PBMC from *M. bovis*-infected animals was found to be dependent upon the presence of $CD4^+$ T

cells as depletion of these cells totally abrogated the IFN-γ production (Walravens, Wellemans et al. 2002). This would suggest that either the $CD8^+$ cells are unable to respond to PPD-B *in vitro* in absence of $CD4^+$ T cells or that the $CD8^+$ cells are reacting in a bystander manner to a cytokine produced by the $CD4^+$ cells Previously some subsets of memory $CD8^+$ T cells have been reported to produce IFN-γ in response to culture with specific cytokines. Walverens and co-workers reported that $CD8^+$ cells isolated from the PBMC from *M. bovis*-infected animals required the addition of IL-2 and either PPD-B or BCG presented by antigen presenting cells (APCs) in order to produce IFN-γ *in vitro* (Walravens, Wellemans et al. 2002). This suggests that the $CD4^+$ T cells may help the $CD8^+$ T cells through the production of IL-2.

$CD8^+$ cells may contribute to the control of *M. bovis* infection in cattle through the inhibition of mycobacterial growth as co-culture of MBSE stimulated PBMC with *M. bovis*-infected Mϕ reduced the rate of mycobacterial metabolism. This inhibition was observed in PBMC from both un-infected and *M. bovis*-infected animals suggesting it may not be antigen specific, however PBMC from infected animals induced greater release of mycobacteria from the infected Mϕ. Furthermore $CD8^+$ T cell lines established from MBSE stimulated PBMC from *M. bovis*-infected animal induced a higher release of mycobacteria from infected cells compared to $CD4^+$ T cells (Liebana, Aranaz et al. 2000).

A possible role for bovine $CD8^+$ T cells in the lysis of infected Mϕ was suggested by Skinner et al who showed that PBMC from *M. bovis*-infected animals stimulated *in vitro* with *M. bovis* displayed cytolytic activity towards both uninfected and infected Mϕ. Depletion of $CD8^+$ cells from the PBMC induced a decrease in the lysis of *M. bovis* infected Mϕ in some of the animals. Although this paper suggests that the $CD8^+$ cells contribute to the detected lysis, it is unclear if this killing is antigen specific as the percentage killing of uninfected Mϕ by the CD8-depleted PBMC is not shown (Skinner, Parlane et al. 2003).

In addition to a protective role in bovine TB, $CD8^+$ cells were implicated to contribute to the pathology observed in *M. bovis*-infected animals. Antibody depletion of $CD8^+$ cells during the first two weeks of infection resulted in a decreased production of IFN-γ by

PBMC stimulated with PPD-B and an reduced lesion score at necropsy compared to control infected animals (Villarreal-Ramos, McAulay et al. 2003).

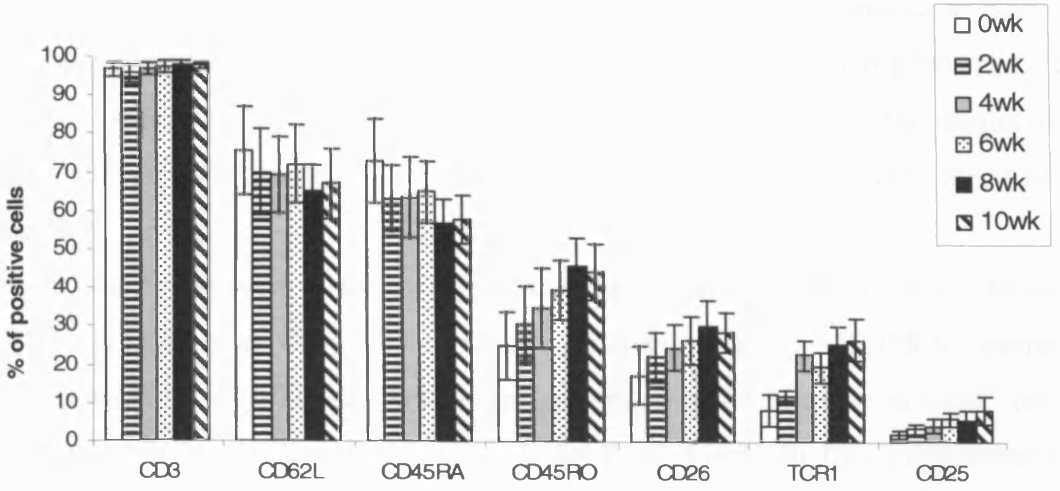
At present the role of $CD8^+$ T cells in immunity to *M. bovis* infection in cattle has been poorly characterised. A clearer understanding of the contribution of different T cell subsets, in particular $CD8^+$ T cells, in mediating protection against *M. bovis* infection is required for design of improved vaccines. In addition, a comparison of immune responses elicited during *M. bovis* infection in animals that are protected against disease and those that develop disease will further our knowledge of protective immune responses. The aim of this chapter is to determine if *M. bovis* infection of cattle induces a $CD8^+$ T cell response and if so, to characterise the role that these cells may play during infection. The objectives of this chapter are to first, investigate the effects of *M. bovis* infection on the proportions of circulating $CD8^+$ T cell subsets. Second, to determine whether mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells can be detected after infection with *M. bovis*. Third, to compare the kinetics and magnitude of the $CD8^{hi}TCR1^-$ T cell responses induced by *M. bovis* infection of BCG-vaccinated and non-vaccinated animals. Fourth, to establish if these $CD8^{hi}TCR1^-$ T cells contribute to the killing of mycobacteria inside Mϕ and finally to explore if *M. bovis*-reactive $CD8^{hi}TCR1^-$ T cells can be detected at the site of infection.

6.2 Immune responses in PBMC from animals infected with *M. bovis*

6.2.1 Changes in the proportion of $CD8^+$ T cell subsets in blood induced by *M. bovis* infection

Infection induces the clonal expansion of antigen specific T cells in the lymph node and the subsequent migration of large numbers of activated T cells through the blood to the site of infection. It is therefore conceivable that a transient increase in the proportion of activated cells present in the blood may occur during *M. bovis* infection. To investigate this, PBMC was isolated from *M. bovis* infected animals and two-colour flow cytometry was used to analyse expression of surface molecules on circulating $CD8^{hi}$ T cells.

A



B

WEEKS	CD26	CD62L	CD45RA	CD3	CD25	TCR1	CD45RO
2 v 0	***		*		**	***	***
4 v 0	***		*		***	***	***
6 v 0	***		*		***	***	***
8 v 0	***	*	***		***	***	***
10 v 0	***	*	***		***	***	***
2 v 4						***	
2 v 6						***	*
2 v 8				*		***	***
2 v 10	*				**	***	***
4 v 6							
4 v 8							***
4 v 10					*		**
6 v 8						**	
6 v 10						***	
8 v 10							

Figure 6.2.1 Changes in the proportion of $CD8^+$ T cell subsets in blood after infection with *M. bovis*. Blood samples were taken from five animals before and at 2 wk intervals post-infection with 10^4 *M. bovis* administered intranasally. PBMC was isolated and stained with antibodies to CD8 together with one of the following CD3, CD63L, CD45RA, CD45RO, CD26, TCR1 and CD25. Flow cytometry was used to gate on the lymphocyte fraction and analyse cells for expression of surface molecules. The mean and standard deviation is shown from five animals. Statistically significant differences in the percentage of CD8hi T cells expressing a particular surface molecule between each timepoint is shown in B. Tukey pairwise comparison was used to determine if differences were significant *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 6.2.1 shows that the percentage of $CD8^{hi}TCR1^{+}$ T cells present in blood increased during *M. bovis* infection with the most significant increase occurring between 2 and 4 wks post-infection. The function of $CD8^{+}$ γδ T cells is unclear but the results indicate that these cells are activated early during infection and migrate through the blood to the site of infection.

Gradual increases were also observed in the percentage of $CD8^{hi}$ T cells in blood that were $CD25^{+}$, $CD26^{+}$ and $CD45RO^{+}$ whereas the percentage of $CD45RA^{+}$ decreased post-infection (Fig 6.2.1). These changes are suggestive of an increase in $CD8^{hi}$ cells in blood with an activated phenotype during *M. bovis* infection. In this experiment it was not investigated whether the increase in the percentage of activated $CD8^{hi}$ T cells was due to increases in activated γδT cells or αβ T cells or both. This experiment does however show that changes in the proportion of activated $CD8^{hi}$ T cells in blood can be detected after infection with *M. bovis*.

6.2.2 CFDA staining of PBMC from unvaccinated/infected animals

To determine whether infection with *M. bovis* results in the induction of mycobacteria-reactive $CD8^{hi}TCR1^{-}$ T cells, CFDA labelled PBMC from two infected animals, 64 and 83, were cultured with ConA, PPD-B or *M. bovis*-infected autologous PBMC. PBMC were harvested and analysed using flow cytometry for expression of CFDA and cell surface markers. Loss of CFDA fluorescence identifies cells that have divided and the number of cell divisions is shown as peaks on a histogram chart. The mitogen ConA induced a high proportion of $CD8^{hi}TCR1^{-}$ T cells from both animals to undergo a number of cell divisions. In both animals, a higher percentage of $CD8^{hi}TCR1^{-}$ T cells proliferated in response to *M. bovis*-infected cells compared to PPD-B (Fig 6.2.2 and 6.2.3). A small percentage of $CD8^{hi}TCR1^{-}$ T cells from animal 83 but not from animal 64 divided in response to PPD-B. A greater percentage of $CD8^{hi}TCR1^{-}$ T cells divided in response to both *M. bovis* and PPD-B at 5 days in culture compared to 3 days (Fig 6.2.2 and 6.2.3).

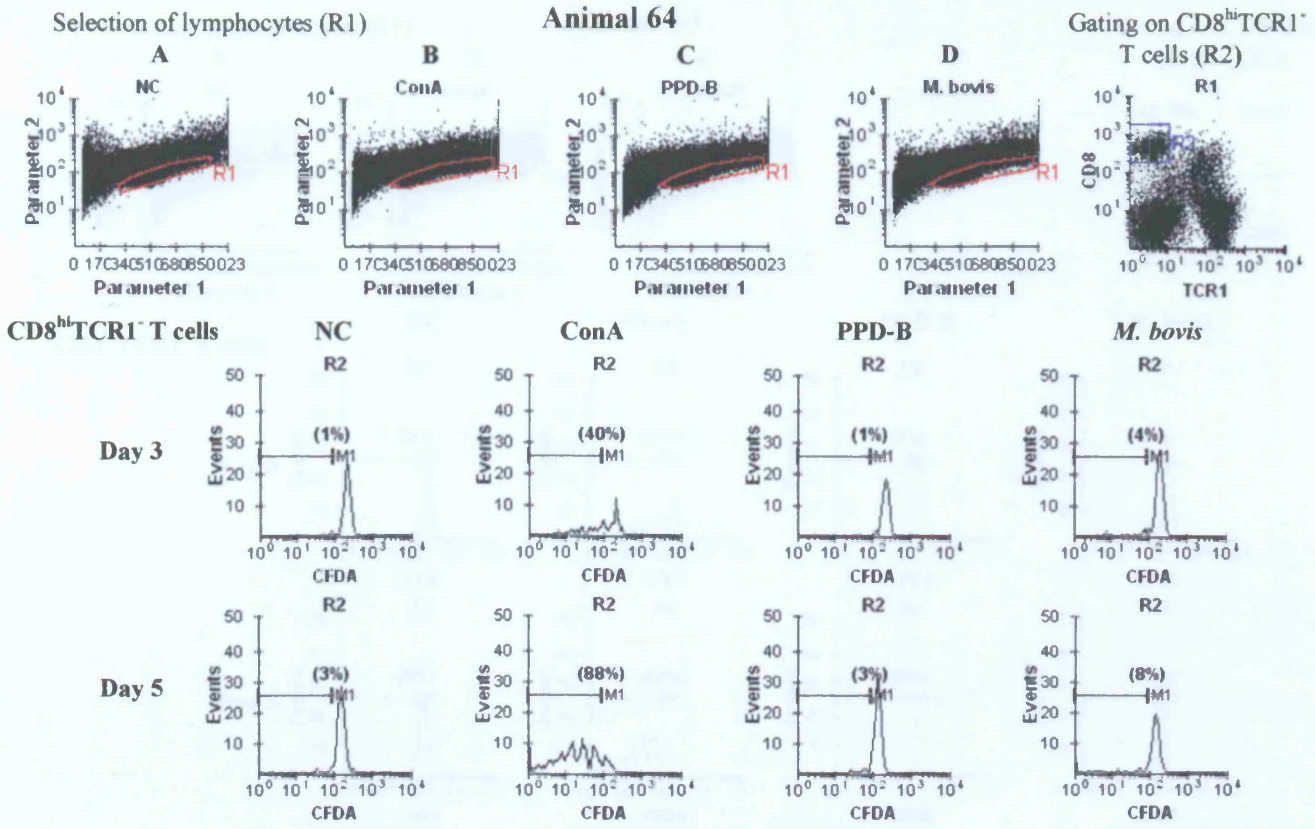


Figure 6.2.2 Proliferation of mycobacteria-reactive CD8^{hi}TCR1^{-} T cells from a *M. bovis* infected animal (64) measured by loss of CFDA. PBMC were isolated from an infected animal at 12 wks post-infection and were labelled with CFDA. PBMC were cultured alone (NC) (A) or with Con A (10μg/ml) (B), PPD-B (10μg/ml) (C), or *M. bovis* (MOI of 5:1) (D), Cells were harvested after 3 and 5 days of culture and washed and stained with antibodies to CD8 and TCR1. A gate was drawn around the resting and activated lymphocytes (R1) and applied to a dotplot showing CD8 and TCR1 and a second gate was drawn to select the CD8^{hi}TCR1^{-} T cells (R2). The number of times the CD8^{hi}TCR1^{-} T cells had divided was measured by a decrease expression of CFDA. Dotplots are shown from one experiment and are representative of two experiments. No staining was observed with the isotype controls.

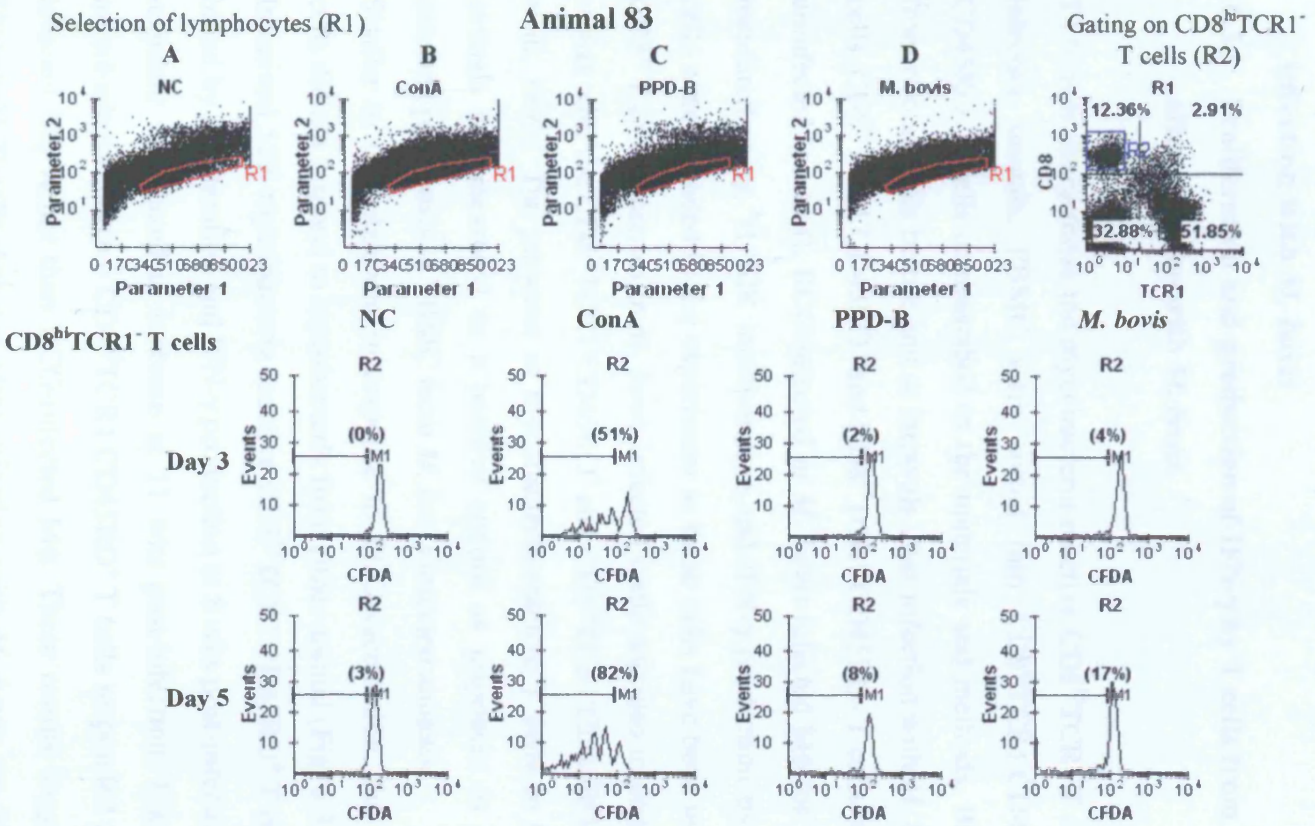


Figure 6.2.3 Proliferation of mycobacteria reactive $CD8^{hi}TCR1^{-}$ T cells from a *M. bovis* infected animal (83) measured by loss of CFDA.
PBMC were isolated from an infected animal and were labelled with CFDA. PBMC were cultured alone (NC) (A) or with Con A (10μg/ml) (B), PPD-B (10μg/ml) (C), or *M. bovis* (MOI of 5:1) (D), Cells were harvested after 3 and 5 days of culture and washed and stained with antibodies to CD8 and TCR1. A gate was drawn around the resting and activated lymphocytes (R1) and applied to a dotplot showing CD8 and TCR1 and a second gate was drawn to select the $CD8^{hi}TCR1^{-}$ T cells (R2). The number of times the $CD8^{hi}TCR1^{-}$ T cells had divided was measured by a decrease expression of CFDA. Dotplots are shown from one experiment and are representative of two experiments. No staining was observed with isotype controls.

6.3 Development of immune responses in non-vaccinated animals after infection with *M. bovis*

6.3.1 Proliferation and production of IFN-γ by T cells from non-vaccinated animals after infection with *M. bovis*

To further characterise the mycobacteria-reactive $CD8^{hi}TCR1^{-}$T cells detected in *M. bovis*-infected animals, PBMC were sorted into $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells as described in the materials and methods. Blood samples were taken from two animals before and at intervals after infection with *M. bovis*. PBMC, total $CD8^{+}$ cells, $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}$CD45RO- T cells were cultured *in vitro* with uninfected (control), BCG-infected or *M. bovis*-infected Mϕ for 5 days. Proliferation was measured using ^{3}H TdR incorporation and IFN-γ production by ELISA. The total $CD8^{+}$ cells were included in the experiment as these cells have been used in previous studies of $CD8^{+}$ T cell responses in *M. bovis* infected cattle and also to compare the response of this subset with the $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells (Liébana, Girvin et al. 1999). The presence of mycobacteria-reactive T cells in PBMC from the infected animals was measured as a positive control as responses to mycobacteria have been detected previously in PBMC from *M. bovis* infected animals.

Similar to that observed in response to BCG-vaccination, the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells did not respond to mycobacteria from either animal (Fig. 6.3.1 and 6.3.2).

In animal 529, mycobacteria reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells were first detected in blood by proliferation and IFN-γ production at 8 wks post-infection. The magnitude of this response was found to increase at 11 wks post-infection. Figure 6.3.1 shows that the mycobacteria reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells responded preferentially to *M. bovis* infected Mϕ rather than BCG-infected Mϕ. These results suggest that the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells detected after infection with *M. bovis* are likely to be responding to antigens that are specific to *M. bovis* and are not expressed or are expressed at a lower level by BCG.

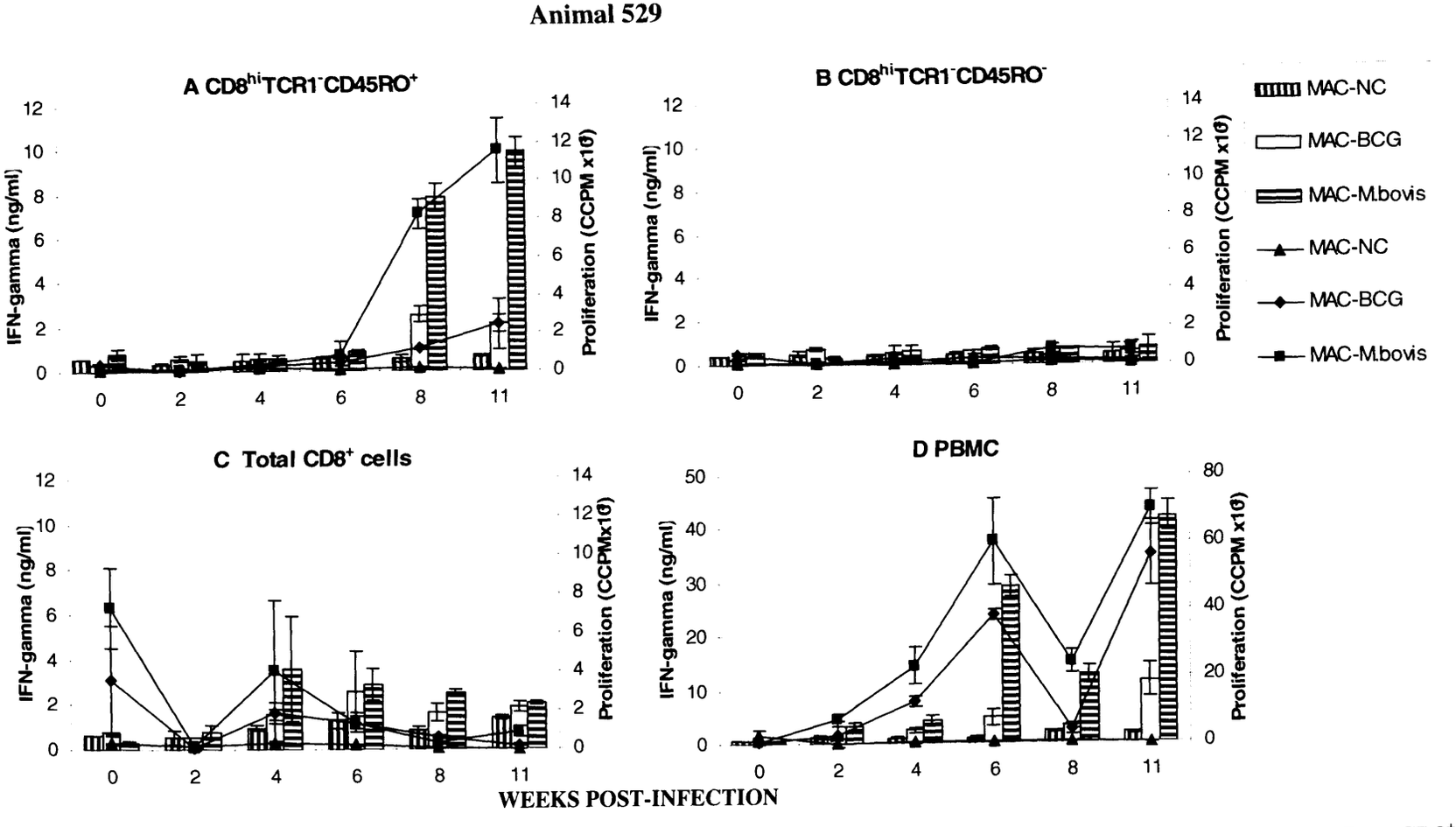


Figure 6.3.1 Proliferation and production of IFN-γ by mycobacteria-reactive T cells that develop after infection with *M. bovis* (529). Total $CD8^+$ cells were isolated from PBMC using MACS isolation system and stained with antibodies to the γδ TCR (TCR1) and CD45RO. Cells were sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$ T cells. The $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected (MAC-NC), BCG-infected (MAC-BCG) or *M. bovis* -infected macrophages (MAC-*M. bovis*). Proliferation was measured by uptake of tritiated thymidine (^3H) displayed on right axis as CCPM (line) and production of IFN-γ was measured by ELISA and is displayed on left axis as ng/ml (column). The mean and standard deviation is shown for triplicate samples.

Prior to infection with *M. bovis* the total $CD8^+$ cells from both animals were found to proliferate in response to BCG and *M. bovis* infected Mϕ. After infection with *M. bovis*, these cells produced IFN-γ in response to control Mϕ, BCG-infected and *M. bovis*-infected Mϕ (Fig. 6.3.1C). The pattern of response to mycobacteria observed in the sorted $CD8^{hi}TCR1^-CD45RO^+$ T cells was very different to that observed in the total $CD8^+$ cells. The low level production of IFN-γ by the total $CD8^+$ cells appears to peak at 4 wks post-infection whereas the response of the $CD8^{hi}TCR1^-CD45RO^+$ T cells was highest at 11 wks post-infection. The results in figure 6.3.1 suggest that it is likely that different cell-types in the $CD8^{hi}TCR1^-CD45RO^+$ T cells and the total $CD8^+$ cells are responding to mycobacteria in this assay.

After infection with *M. bovis*, a response to mycobacteria was detected in the PBMC at 4 wks post-infection, which peaked at 6 and 11 wk post-infection (Fig 6.3.1D). The PBMC proliferated in response to BCG and *M. bovis*-infected Mϕ whereas production of IFN-γ was observed predominantly in response to *M. bovis* infected Mϕ. The responsiveness to both BCG and *M. bovis* suggests that cells in the PBMC recognise antigens shared between the two mycobacteria predominantly proliferate whereas the cells recognising *M. bovis* specific antigens have also acquired the ability to produce IFN-γ.

The level of proliferation and production of IFN-γ by the PBMC was of a much greater magnitude than that observed by the $CD8^{hi}TCR1^-CD45RO^+$ T cells and total $CD8^+$ cells. The kinetics of the response to mycobacteria detected in the PBMC also differs from that observed in the $CD8^{hi}TCR1^-CD45RO^+$ T cells and total $CD8^+$ cells. It has been shown that $CD4^+$ T cells from infected animals proliferated at a higher rate than $CD8^+$ T cells in response to mycobacteria (Liébana, Girvin et al. 1999). In addition, changes in the proportion of $CD4^+$ T cells in blood have been reported after infection with *M. bovis* at an earlier stage of infection compared to changes observed in the proportion of $CD8^+$ T cells (Pollock, Pollock et al. 1996). It is likely that response detected in the PBMC may be attributed to mycobacteria reactive $CD4^+$ T cells.

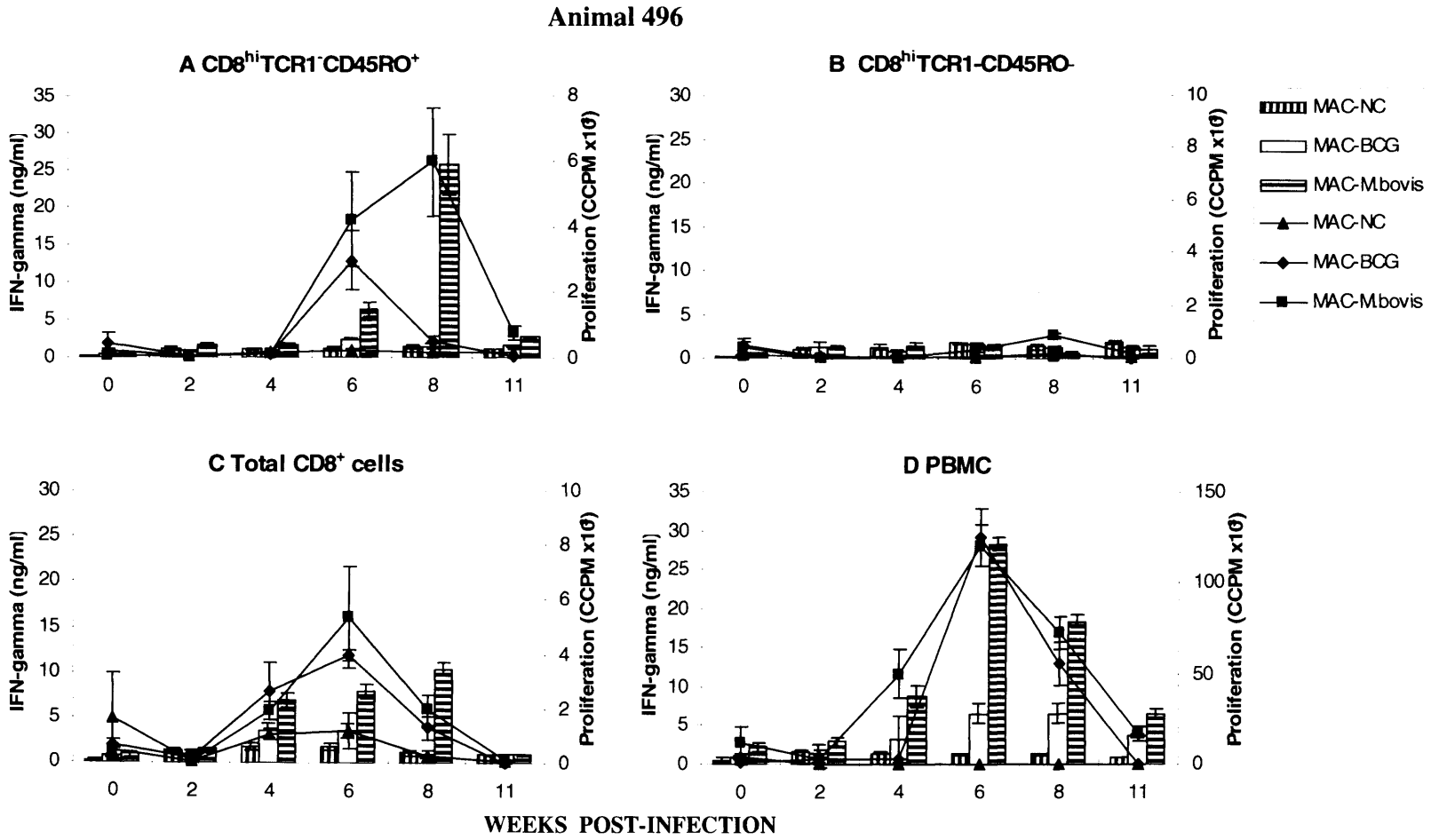


Figure 6.3.2. Proliferation and production of IFN-γ by mycobacteria-reactive T cells after infection with *M. bovis* (496). Total $CD8^+$ cells were isolated from PBMC using MACS isolation system and stained with antibodies to the γδ TCR (TCR1) and CD45RO. Cells were sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$ T cells. $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected (MAC-NC), BCG-infected (MAC-BCG) or *M. bovis* -infected macrophages (MAC-*M. bovis*). Proliferation was measured by uptake of ^3H TdR displayed on right axis as CCPM (line) and production of IFN-γ was measured using ELISA and is displayed on left axis as ng/ml (column). The mean and standard deviation is shown for triplicate samples.

In response to infection with *M. bovis*, mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells were detected in blood from animals 496 at 6 wks post-infection (Fig. 6.3.2A) These cells proliferated and produced IFN-γ in response to culture with *M. bovis*-infected Mϕ and this response peaked at 8 wks post-infection. At 11 wks post-infection no mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells were detected in blood from animal 496.
In animal 496, similar to animal 529, the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cell responded mainly to *M. bovis* infected Mϕ, with the exception of 6 wk post-infection at which the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animal 496 also proliferated in response to BCG-infected Mϕ.

Again in animal 496, similar to animal 529, the pattern of response to mycobacteria observed in the total $CD8^{+}$ cells and the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells are shown in figure 6.3.2 to be different. The mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells could be detected at 6 wks post-infection whilst the total $CD8^{+}$ cells responded to mycobacteria at 4 wks. In addition, the responding $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells proliferated to a greater extent and produced more IFN-γ than the total $CD8^{+}$ cells.
After infection with *M. bovis*, mycobacteria-reactive cells present in the PBMC from animal 496 were first detected at 4 wks post-infection and the measured response was highest at 6 wks post-infection (Fig 6.3.2D). Whereas the level of proliferation detected was similar after culture with BCG or *M. bovis* infected Mϕ, a greater amount of IFN-γ was produced in response to *M. bovis* compared to BCG. The magnitude of the response observed by PBMC is again much higher than that observed in the total $CD8^{+}$ T cells and the $CD8^{hi}TCR1^{-}CD45RO^{+}$ in animal 496 (Fig 6.3.2).
Analysis of immune responses to mycobacteria from uninfected age-matched animals demonstrated that IFN-γ production could be detected in the total $CD8^{+}$ and PBMC after culture with both BCG and *M. bovis*-infected Mϕ (Fig 6.3.3). It is possible that this response is antigen specific, as these animals may have been sensitised to mycobacteria by exposure to environmental mycobacteria such as *M. avium*. It is also possible that innate immune cells present in the total $CD8^{+}$ cells and PBMC are responding to the

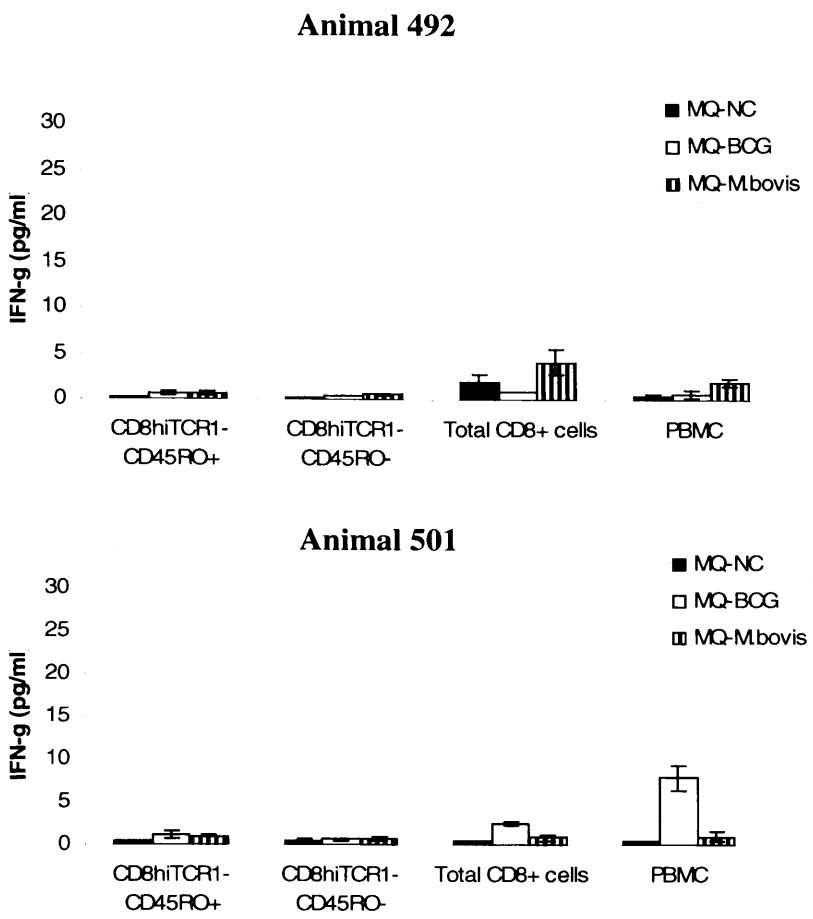


Figure 6.3.3. Production of IFN-γ by T cells from uninfected age-matched animals 492 and 501. $CD8^+$ cells were isolated from PBMC using MACS isolation system. Cells were stained with antibodies to the γδ TCR (TCR1) and CD45RO and sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), unsorted $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected macrophages or macrophages infected with BCG, *M. avium* or *M. bovis* (MAC). Proliferation was measured by uptake of ^3H TdR shown as CCPM (line) and production of IFN-γ was measured by ELISA and shown as ng/ml (column). The mean and standard deviation is shown for triplicate samples.

mycobacteria. No response that was significantly higher than the background levels to uninfected Mϕ could be detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ and $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells from the control animals after culture with mycobacteria (Fig 6.3.3). This suggests that the responses to mycobacteria observed in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from the infected animals are likely to have been induced by infection with *M. bovis*.

6.4 Development of immune responses in BCG-vaccinated animals after infection with *M. bovis*

6.4.1 Proliferation and production of IFN-γ by T cells from BCG-vaccinated after infection with *M. bovis*

At present there is no information regarding the development of $CD8^{+}$ T cell responses in BCG vaccinated animals after infection with *M. bovis*. Therefore to investigate this, three animals 23, 29 and 34 that had been vaccinated with BCG as neonates, described in chapter 5, were infected with *M. bovis* at 9 wks post-vaccination. The $CD8^{hi}TCR1^{-}CD45RO^{+}$, $CD8^{hi}TCR1^{-}CD45RO^{-}$, total $CD8^{+}$ cells and PBMC were isolated and stimulated as described in materials and methods. Proliferation was measured using ^{3}H TdR incorporation and IFN-γ production by ELISA and these techniques were used to evaluate T cell responses induced by *M. bovis* infection.

To recap, it was shown in chapter 5 that prior to BCG vaccination, mycobacteria-reactive T cells were present in the blood of the neonatal animals, this is shown in figures 6.4.1, 6.4.2 and 6.4.3 as prevacc. BCG vaccination induced immune responses in the three animals which peaked at between 7-9 wks post-vaccination. Therefore at the time of infection, there are T cells present in the blood that respond highly to mycobacteria. The animals were infected with *M. bovis* and blood samples were taken at 5, 7, 9 and 11 wks post-challenge. In figures 6.4.1-3, the proliferation and IFN-γ production detected prior to challenge in these young animals is considerably higher than observed post-challenge. Nonetheless the responses detected post-challenge are sizable compared to the responses detected in the non-vaccinated infected animals. It is possible that the mycobacteria are modulating the immune responses of these animals.

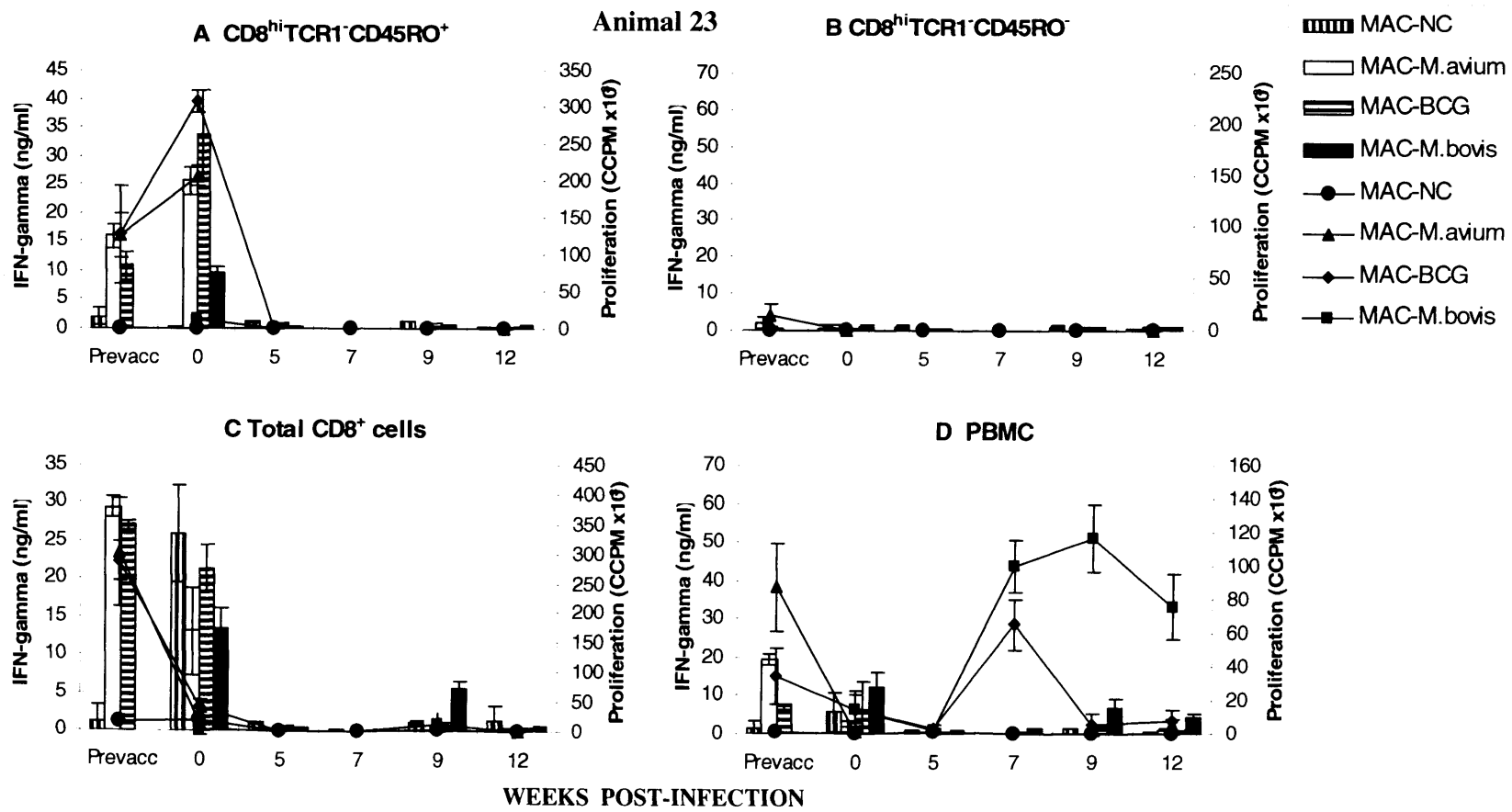


Figure 6.4.1. Proliferation and production of IFN-γ by T cells from an *M. bovis* -infected BCG vaccinated animal (23). $CD8^+$ cells were isolated from PBMC from an animal vaccinated with BCG and infected with *M. bovis* at 9 wks post-vacc (0wk) using MACS isolation beads. Cells were stained with antibodies to the γδ TCR (TCR1) and CD45RO and sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^-CD45RO^-$ (B), total $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected (MAC-NC), BCG-infected (MAC-BCG) or M. *bovis*-infected macrophages (MAC- *M. bovis*). Proliferation was measured by uptake of tritiated thymidine (^3H) displayed on right axis as CCPM (line) and production of IFN-γ was measured using ELISA and is displayed on left axis as ng/ml (column). The mean and standard deviation is shown for triplicate samples

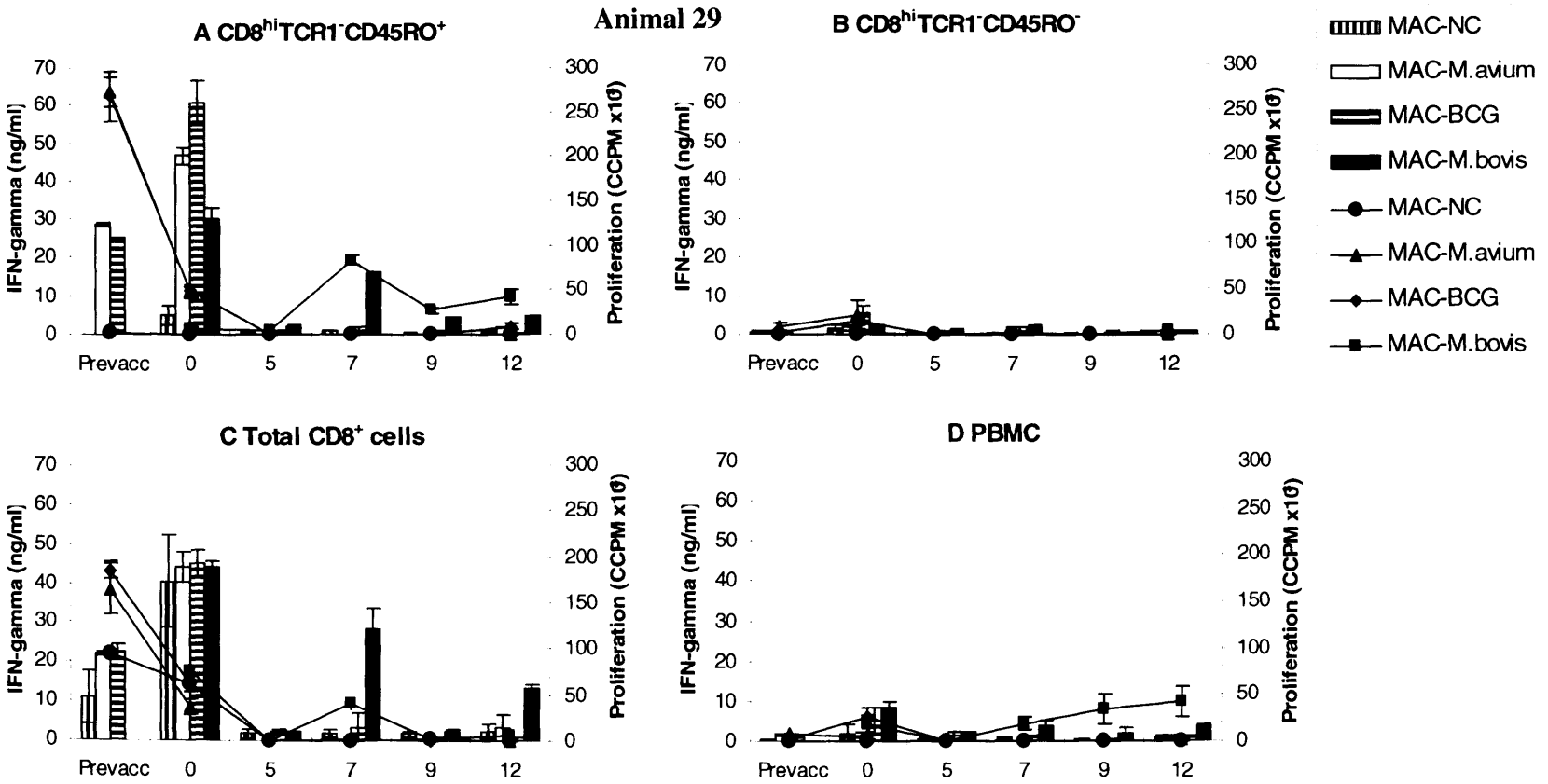


Figure 6.4.2. Proliferation and production of IFN-γ by T cells from an *M. bovis* -infected BCG vaccinated animal (29). $CD8^+$ cells were isolated from PBMC from an animal vaccinated with BCG and infected with *M. bovis* at 9 wks post-vacc (0wk) using MACS isolation beads. Cells were stained with antibodies to the γδ TCR (TCR1) and CD45RO and sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^-CD45RO^-$ T cells (B), unsorted $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected macrophages or macrophages infected with BCG, *M. avium* or *M. bovis* (MAC). Proliferation was measured by uptake of tritiated thymidine (^3H) displayed on right axis as CCPM (line) and production of IFN-γ was measured using ELISA and is displayed on left axis as ng/ml (column). The mean and standard deviation is shown for triplicate samples

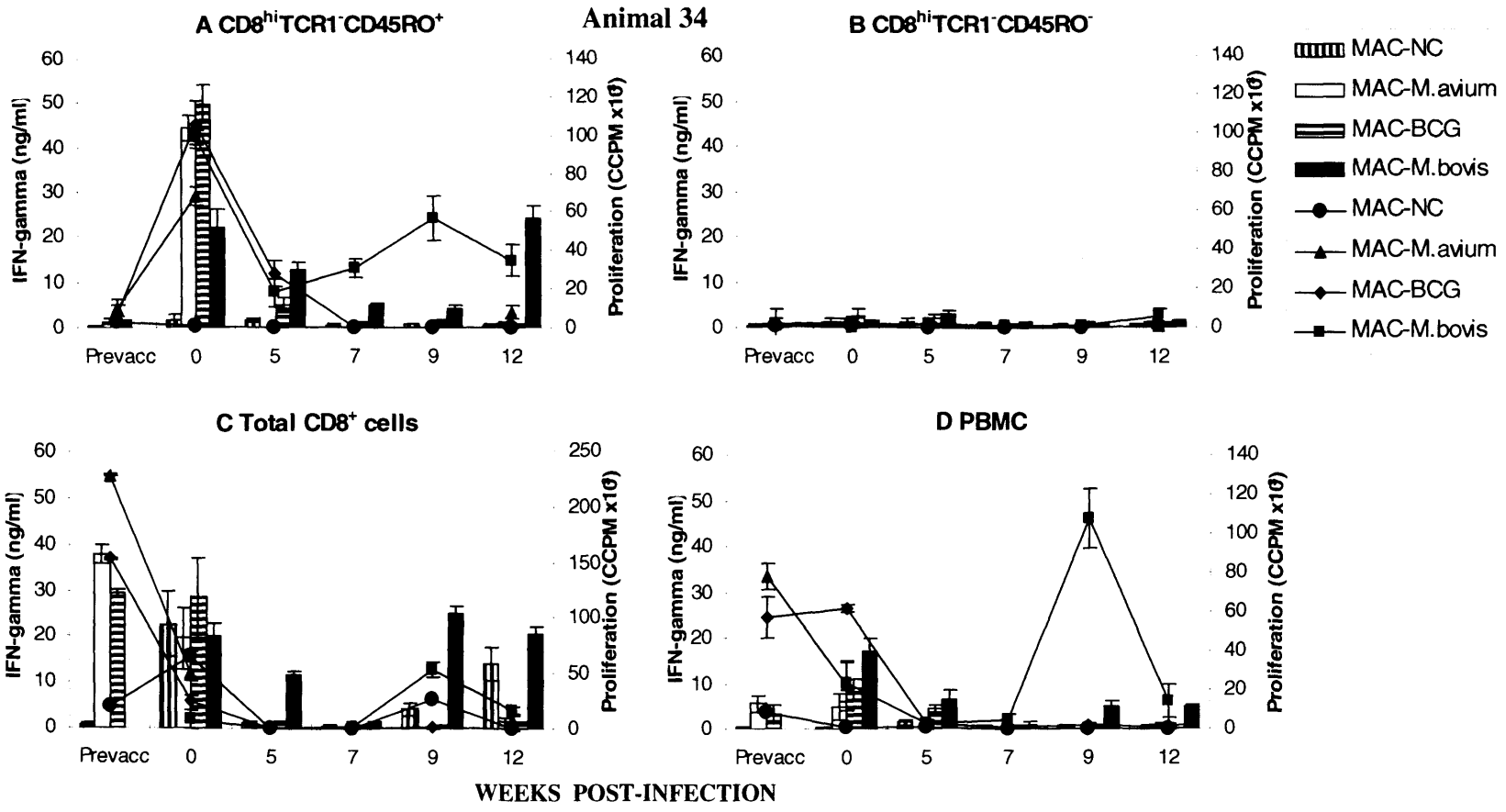


Figure 6.4.3. Proliferation and production of IFN-γ by T cells from an *M. bovis*-infected BCG vaccinated animal (34). $CD8^+$ cells were isolated from PBMC from an animal vaccinated with BCG and infected with *M. bovis* at 9 wks post-vacc (0wk) using MACS isolation beads. Cells were stained with antibodies to the γδ TCR (TCR1) and CD45RO and sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^-CD45RO^-$ T cells (B), unsorted $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected macrophages or macrophages infected with BCG, *M. bovis* or *M. bovis* (MAC). Proliferation was measured by uptake of tritiated thymidine (^3H) displayed on right axis as CCPM (line) and production of IFN-γ was measured using ELISA and is displayed on left axis as ng/ml (column). The mean and standard deviation is shown for triplicate samples

In animal 23, no mycobacteria-reactive cells were detected in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ or the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells after infection with *M. bovis* (Fig. 6.4.1A and B). However, mycobacteria reactive cells in the PBMC from animal 23 were detected at 7 wks post-infection. These cells proliferated highly in response to *M. bovis* infected Mϕ. These results suggest that mycobacteria-reactive $CD4^{+}$ T cells are present in the PBMC from animal 23 (Fig 6.4.1D).

In animal 29, memory mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells induced by BCG vaccination were not be detected in blood after infection with *M. bovis*. However, *M. bovis*-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells were detected at 7 wks post-infection. These cells proliferated and produced IFN-γ after culture with *M. bovis* infected Mϕ but not with BCG or *M. avium*-infected Mϕ. A similar pattern of response in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells was detected in the total $CD8^{+}$ cells from animal 29 to *M. bovis* infected Mϕ (Fig 6.4.2). In addition, mycobacteria-reactive cells were detected in the PBMC from animal 29 at 7 wks post-infection. These cells proliferated and produced a low level of IFN-γ in response to culture with *M. bovis* infected Mϕ.

Apart from a modest transient response to BCG-infected Mϕ detected at 5 wks post-challenge, the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animal 34 responded only to *M. bovis* infected Mϕ post-infection (Fig. 6.4.3). The proliferative response of these cells peaked at 9 wks post-infection whereas the production of IFN-γ peaked at 12 wks post-infection. These results suggest that perhaps the proliferation of the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells generated effector cells that produce IFN-γ.
The total $CD8^{+}$ cells from animal 34 are found to respond to both uninfected and *M. bovis* infected Mϕ at 9 and 12 wks post-challenge (Fig 6.4.3). After infection with *M. bovis* a transient proliferative response was observed at 9 wks post-infection in the PBMC from animal 34. In addition, a low level production of IFN-γ was also detected in the PBMC in response to *M. bovis*-infected Mϕ at 5, 9 and 12 wks post-infection (Fig 6.4.3). Prior to vaccination with BCG, mycobacteria-reactive cells were detected in the PBMC.

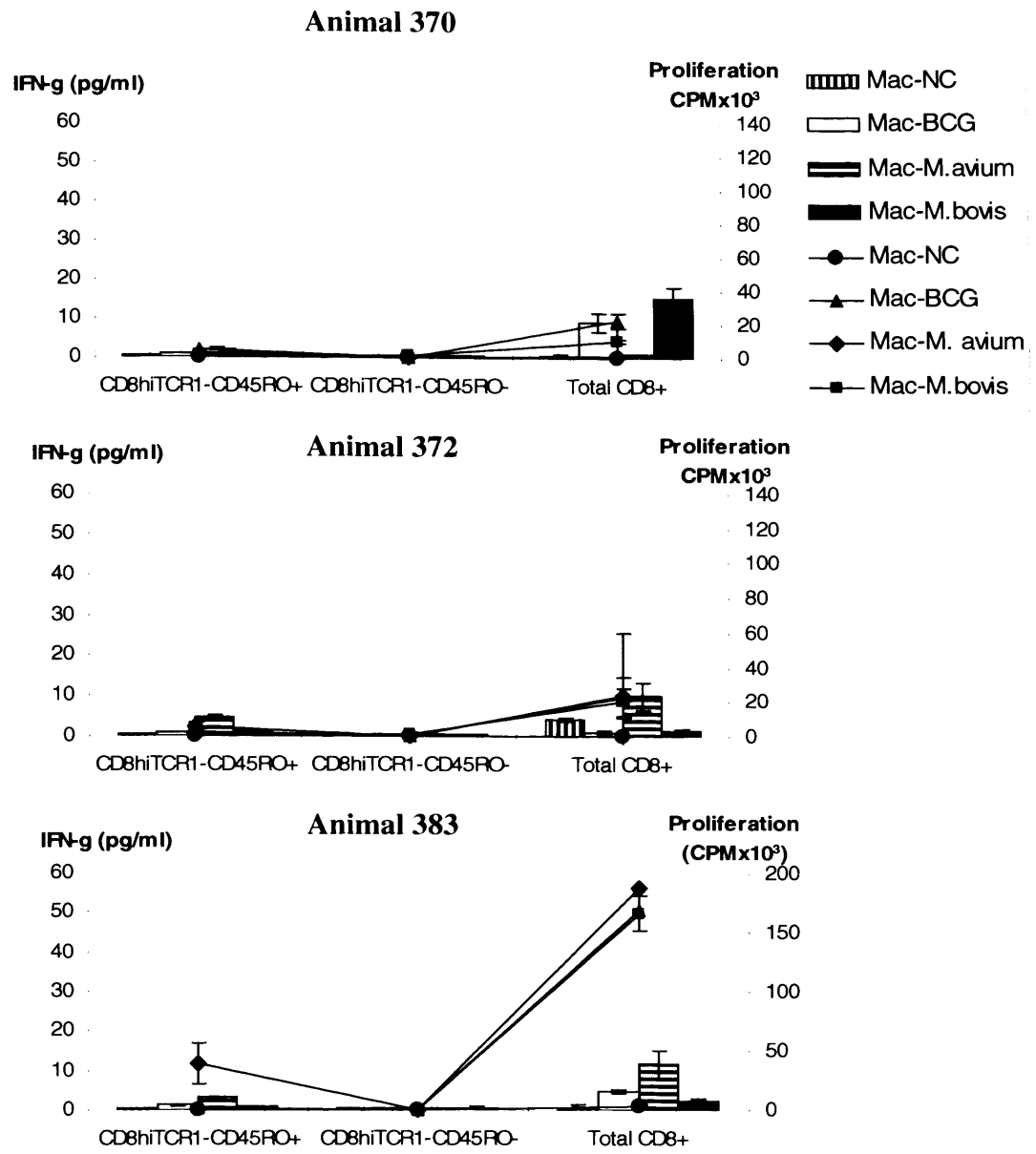


Figure 6.4.4. Proliferation and production of IFN-γ by T cells from age-matched uninfected animals after culture with mycobacteria. $CD8^+$ cells were isolated from PBMC from control animals using MACS isolation system. Cells were stained with antibodies to the γδ TCR (TCR1) and CD45RO and sorted on a MoFlo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$. The $CD8^{hi}TCR1^-CD45RO^+$ (A) and $CD8^{hi}TCR1^+CD45RO^-$ (B), unsorted $CD8^+$cells (C) and PBMC (D) were cultured for 5 days with uninfected macrophages or macrophages infected with BCG, *M. avium* or *M. bovis* (MAC). Proliferation was measured by uptake of ^3H TdR shown as CCPM (line) and production of IFN-γ was measured by ELISA and shown as ng/ml (column). The mean and standard deviation is shown for triplicate samples.

It was found that vaccination with BCG boosted this response but may not have induced a strong memory response, which may, in part, explain the low level responses observed in the PBMC after infection with *M. bovis*.

The response of T cells to mycobacteria from age-matched uninfected animals (12 wks old) was also investigated. Figure 6.4.4 shows that a modest response to *M. avium* was detected in $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from one of the three animals. This suggests that this particular animal may have been exposed to environmental mycobacteria. Mycobacteria-reactive cells were also detected in the total $CD8^{+}$ cells from all three animals (Fig 6.4.4).

The mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells induced by BCG vaccination prior to infection may have migrated to the site of infection and therefore their frequency in blood declined to levels below detection by this assay. It was not possible to investigate a time-point earlier than 5 wks post-infection, it is possible that the memory $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells induced by vaccination may have responded earlier than this timepoint. This suggests that the memory T cells induced by BCG vaccination that respond to BCG, *M. avium* or *M. bovis*-infected Mϕ are not present in the blood at a detectable frequency after infection with *M. bovis*. It is likely that the response in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells detected post-challenge is the result of a primary response to *M. bovis* and is specific for antigens expressed by *M. bovis* and not by BCG or *M. avium*.

6.4.2 Effect of $CD8^{+}$ T cells from BCG-vaccinated animals infected with *M. bovis* on the survival of intracellular mycobacteria

Mycobacteria-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells were found to develop after infection with *M. bovis*. These cells proliferated and produced IFN-γ after culture with *M. bovis* infected Mϕ. To further define the role of these $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells, the effect of co-culture with infected Mϕ on the viability of the mycobacteria was investigated. Mϕ infected with either BCG or *M. bovis*, were cultured alone or with $CD8^{hi}TCR1^{-}$ $CD45RO^{+}$,

$CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells or total $CD8^{+}$ cells for 5 days. The cells were then lysed and serial dilutions were made from the supernatants in 7H9 media and plated onto 7H10 (BCG) or 7H11 (*M. bovis*) plates to determine mycobacterial colony forming units (cfu). The number of mycobacteria that had been taken up by the Mϕ was calculated prior to co-culture with T cells. Table 6.4.1 shows mycobacterial cfu per 10^6 Mϕ and the percentage decrease in cfu of mycobacteria within Mϕ after culture with T cells compared to infected Mϕ cultured alone is shown in brackets. The infection rates of each mycobacteria was found to be similar in both animals although Mϕ from animal 23 contained a slightly higher cfu of both BCG and *M. bovis*.

The cfu of the BCG present in the infected Mϕ from both animals after 5 days in culture decreased dramatically (Table 6.4.1). In addition, the cfu of *M. bovis* within Mϕ was also found to decrease but to a lesser extent than the BCG. This observed difference in survival rates may be related to differences in the virulence of the two mycobacteria as the BCG may be more susceptible to killing by the Mϕ. A further reduction in bacterial cfu was observed in BCG-infected Mϕ cultured with $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells, $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells and total $CD8^{+}$ cells from animal 23.

In animal 34 a large reduction in BCG cfu was observed after culture of BCG-infected Mϕ with $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells. While a much smaller reduction was found after culture with total $CD8^{+}$ cells and no difference was observed in BCG cfu after culture with $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells (Table 6.4.1).

A similar decrease in *M. bovis* cfu was observed after *M. bovis*-infected Mϕ were cultured with either $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells or total $CD8^{+}$ cells from animal 23. In contrast to BCG, no inhibition of *M. bovis* cfu was observed after culture with the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells from animal 23.

In animal 34, a reduction in *M. bovis* cfu was observed after culture of infected Mϕ with $CD8^{hi}TCR1^{-}CD45RO^{+}$, $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells and total $CD8^{+}$ cells (Table 6.4.1). Consistently, $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from both animals induced the greatest decrease in mycobacterial cfu within Mϕ. Compared to animal 23, the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animal 34 induced a greater decrease in *M. bovis* counts. This difference may reflect the fact that the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from animal 34

	BCG counts ($x10^3$)		*M. bovis* counts ($x10^4$)	
	Animal 23	**Animal 34**	**Animal 23**	**Animal 34**
Infected Mϕ (before culture)	**182** ± 2.2	**145** ± 0.98	**10** ± 0.56	**8** ± 0.28
Infected Mϕ Cultured alone	**5.1** ± 0.8	**1.6** ± 0.2	**4.5** ± 0.3	**2.3** ± 0.09
Infected Mϕ + $CD8^{hi}TCR1^-$ $CD45RO^+$	**1.6** ± 0.3 (69%)	**0.6** ± 0.1 (63%)	**3.3** ± 0.1 (27%)	**1.0** ± 0.1 (57%)
Infected Mϕ + $CD8^{hi}TCR1^-$ $CD45RO^-$	**2.3** ± 0.1 (55%)	**1.7** ± 0.2	**4.6** ± 0.2	**1.9** ± 0.1 (28%)
Infected Mϕ + Total $CD8^+$ cells	**2.8** ± 0.2 (46%)	**1.5** ± 0.2 (6%)	**3.3** ± 0.4 (27%)	**1.5** ± 0.1 (35%)

Table 6.4.1 Effect of $CD8^+$ T cells from vaccinated and challenged animals on survival of mycobacteria within Mϕ. $CD8^+$ cells were isolated from blood using MACS sorting system and stained with antibodies to TCR1 and CD45RO before being sorted on a Moflo cell sorter into $CD8^{hi}TCR1^-CD45RO^+$ and $CD8^{hi}TCR1^-CD45RO^-$ T cells. The sorted cells and total $CD8^+$ cells were cultured with Mϕ infected with either BCG or *M. bovis*. After 5 days the cells were lysed with 0.1% triton X and serial diluations were made in 7H9 media from the supernatant. The dilutions were plated out in triplicate on 7H10 plates for BCG and 7H11 plates for *M. bovis*. After 3 wks in culture the bacterial colonies present on the plates were enumerated. The median and standard deviations are shown for the bacterial counts and the percentage reduction in mycobacterial numbers present when macrophages were cultured with T cells compared to macrophages cultured alone is shown in brackets.

proliferated and produced IFN-γ after culture with *M. bovis*-infected Mϕ whereas no response was detected in these cells post-infection from animal 23.

6.4.3 Comparison of lesion scores in either BCG vaccinated and non-vaccinated animals infected with *M. bovis*

To determine whether the development of $CD8^+$ T cell responses after infection with *M. bovis* could be related to protection against disease, the lesion score of the BCG-vaccinated and non-vaccinated animals was compared. Table 6.4.2 shows that at the time of necropsy, the BCG-vaccinated animals had a low lesion score and the lesions were contained within one tissue. In contrast the non-vaccinated animal 496 had a much higher lesion score and lesions were found in a number of tissues. This is characteristic of a non-vaccinated animal after infection with *M. bovis* as shown by the group mean lesion score of the other non-vaccinated animals. However, supprisingly the non-vaccinated animal 529 had a low lesion score similar to the BCG-vaccinated animals suggesting that the immune response elicited in this animal provided a level of protection against *M. bovis* infection (Table 6.4.2).

	BCG-vaccinated *M. bovis*-infected animals			**Non-vaccinated *M. bovis* infected animals**		
	123	129	134	496	529	Group mean
Lesion score	1	2	2	14	2	19
No. tissue affected	1	1	1	5	1	6

Table 6.4.2 Comparison of disease severity after infection with *M. bovis* in BCG-vaccinated and non-vaccinated animals. At necropsy, lymph nodes and lungs were removed and analysed for the presence of TB lesions. The number of lesions and tissues containing lesions in the BCG-vaccinated and non-vaccinated animals was recorded.

6.5 $CD8^{hi}TCR1^{-}$ T cell responses in the BAL from *M. bovis*-infected animals

Studies investigating the response of $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells in blood to mycobacteria have indicated that *M. bovis*-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells develop after infection with *M. bovis*. However, it is not known if mycobacteria-reactive $CD8^{+}$ T cells migrate to the site of infection. Therefore, the presence of these cells in the lungs of *M. bovis*-infected animals was investigated. BAL was isolated at necropsy of non-vaccinated *M. bovis*-infected animals and was cultured overnight with medium alone, BCG or *M. bovis*. To detect expression of intracellular cytokines, BFA was added for the last 5 hrs of culture. Cells were then washed and analysed for expression of cell surface molecules and intracellular IFN-γ using flow cytometry.

Table 6.5.1 shows that $CD8^{hi}TCR1^{-}$ T cells present in the BAL from the three *M. bovis* infected animals expressed IFN-γ after culture with either BCG or *M. bovis*. A higher percentage of $CD8^{hi}TCR1^{-}$ T cells expressed IFN-γ after culture with *M. bovis* compared to BCG. Furthermore a higher percentage of IFN-γ expressing $CD8^{hi}TCR1^{-}$ T cells were observed in BAL from animal 734 compared to animals 496 and 529.

It is thought that this difference may be related to different route of infection as animals 529 and 496 were inoculated intranasally whereas animal 734 was inoculated intra-tracheally. Intranasal administration of *M. bovis* has been shown to induce disease mainly in the upper respiratory tract and head nodes, whereas intra-tracheal infection with *M. bovis* induces disease predominantly affecting the lung and BALT.

Animal No.	Medium alone		BCG-stimulated		*M. bovis*-stimulated	
	Isotype control	IFN-γ^+ $CD8^{hi}TCR1^-$ T cells	Isotype control	IFN-γ^+ $CD8^{hi}TCR1^-$ T cells	Isotype control	IFN-γ^+ $CD8^{hi}TCR1^-$ T cells
496	0	0	0	4.3 ± 1.4	0	7.4 ± 1.3
529	0	0.6 ± 0.3	0	5.8 ± 1.1	0	7.9 ± 1.9
734	0	1.2 ± 0.4	0	17.9 ± 3.6	0	19.5 ± 2.5

Table 6.5.1 Expression of IFN-γ by $CD8^{hi}TCR1^-$ T cells present in BAL from *M. bovis*-infected animals. At necropsy lungs were removed from *M. bovis*-infected animals and washed with PBS to obtain the BAL. Cells in the BAL were cultured overnight with media, BCG or *M. bovis*. Brefeldin A was added for the final 5hrs of culture. Expression of IFN-γ and cell surface markers was analysed by flow cytometry. The mean percentage for triplicate samples ± SD from three animals is shown.

6.6 Discussion

In this chapter the development of $CD8^+$ T cell responses after infection with *M. bovis* was investigated in non-vaccinated and BCG-vaccinated animals. Previous studies have demonstrated that mycobacterial-reactive $CD8^+$ cells arise during *M. bovis*-infection. However, in past studies the total $CD8^+$ population, was used to analyse $CD8^+$ T cells responses and it was shown in chapter 2 that this population is comprised of NK cells, γδ T cells and αβ T cells. In past studies the responding cell type has not been defined, therefore these studies may have been measuring responses of $CD8^+$ αβ T cells, γδ T cells or NK (Liébana, Girvin et al. 1999; Smyth, Welsh et al. 2001; Denis, Keen et al. 2006). This is the first study to sort the total $CD8^+$ population and compare responses between the total $CD8^+$ cells and $CD8^{hi}TCR1^-CD3^+$ T cells after infection with *M. bovis*.

Initial experiments identified that changes in the circulating population of $CD8^+$ cells could be detected after *M. bovis* infection. The results show that within the first few weeks of infection the percentage of $CD8^{hi}$γδ T cells in blood significantly increased. It has been reported in cattle that percentage of circulating $WC1^+$ γδ T cells increases during the first 2-3 wks post-infection, which was followed by an increase in $CD4^+$ T cells and then latter the $CD8^+$ T cell population increased (Pollock, Pollock et al. 1996). The relationship between $CD8^{hi}$ and $WC1^+$ γδ T cells is unclear as expression of these molecules is mutually exclusive. Expression of WC1 and CD8 defines two subsets of γδ T cells with potentially different functions (MacHugh, Mburu et al. 1997). In bovine TB, γδ T cells have been reported to exhibit both anti-microbial effector functions and an inhibitory/regulatory role (Rhodes, Hewinson et al. 2001).

The percentage of activated $CD8^{hi}$ cells in blood increased during infection. This was demonstrated by an up-regulation in expression of CD25, the IL-2R α-chain, and the memory marker CD45RO. Interestingly, an increase in the proportion of $CD8^{hi}$ cells expressing CD26 was observed with infection. CD26 is an ecto-enzyme which has a number of functions that includes the cleavage of chemokines; therefore an increase in the expression of this molecule is suggestive of an increase in the number of cells that are migrating along chemokine gradients to the site of infection.

In this preliminary experiment the $CD8^{hi}TCR1^{+}$ γδ T cells were not removed for analysis of the surface phenotype of $CD8^{hi}$ T cells. Therefore, this experiment should be repeated using 3 or 4 colour flow cytometry in order to directly analyse $CD8^{hi}TCR1^{-}$ T cell. To substantiate these results analysis of the surface phenotype on $CD8^{hi}$ T cells from age-matched controls is also required to show that the changes observed are induced by infection. It is unlikely that the noted changes are due to the increase in age of the animals over the 10 wk experiment, as the animals were 6 mths when infected and 8 mths at the end of the experiment. From the previous phenotyping results no significant differences were detected in the surface phenotype of $CD8^{hi}TCR1^{-}$ T cells from 6 mth and 8 mth old animals. It would also be of interest to investigate whether the changes in percentages reflect differences in absolute numbers of activated $CD8^{hi}TCR1^{-}$ T cells after infection with *M. bovis*.

Previously, $CD8^{+}$ cells from *M. bovis*-infected animals have been shown to proliferate and produce IFN-γ, after culture with PPD-B, MBSE or *M. bovis* (Liébana, Girvin et al. 1999). In this study $CD8^{hi}TCR1^{-}$ T cells present in PBMC from two *M. bovis* infected animals divided after culture with *M. bovis*-infected Mϕ. A higher percentage of cells divided when cultured with *M. bovis* for 5 days compared to 3 days. This may be due to the presence of subsets of mycobacteria-reactive $CD8^{hi}TCR1^{-}$ T cells with different activation thresholds or reflect $CD8^{hi}TCR1^{-}$ T cells that have undergone a number of cell divisions. In this experiment, a small percentage of $CD8^{hi}TCR1^{-}$ T cells present in the PBMC from one animal divided after culture with PPD-B. In contrast, mycobacteria-reactive $CD8^{hi}TCR1^{-}$ T cells induced by BCG vaccination, described in chapter 5, did not respond to culture with PPD-B. However, in the vaccination experiments, the $CD8^{hi}TCR1^{-}$ T cells were sorted from PBMC and cultured with PPD-B pulsed Mϕ whereas in the study of infected animals, whole PBMC was stimulated with PPD-B. A previous study using PBMC from infected animals showed that IFN-γ production by $CD8^{+}$ T cells in response to PPD-B required the presence of $CD4^{+}$ T cells (Walravens, Wellemans et al. 2002). These results suggest that the ability of the $CD8^{+}$ cells to respond to PPD-B is dependent on the presence of the $CD4^{+}$ T cells and may require $CD4^{+}$ help. PPD-B is a soluble antigen and would typically be processed and presented via MHC class II pathway. Presentation of PPD-B on MHC class I molecules may occur through the direct binding of a peptide as PPD-B comprised of

highly degraded proteins. Alternatively, the PPD-B could be taken up by phagocytosis and during the acidification process peptides from the degraded protein may directly bind to MHC molecules that are either present on the membrane of the phagosome or through a process of macropinocytosis where the PPD-B will enter an alternative MHC class I pathway (Norbury, Hewlett et al. 1995; Norbury, Chambers et al. 1997). Another possibility is that PPD-B is being presented to the $CD8^+$ T cells in the PBMC on non-classical MHC class I molecules such as CD1 (Mazzaccaro, Gedde et al. 1996; Lalvani, Brookes et al. 1998; Canaday, Ziebold et al. 1999).

It is also possible that the $CD8^+$ cells in the PBMC are responding to PPD-B in an antigen-independent manner i.e. to a cytokine produced by the responding $CD4^+$ T cells. Bystander activation of $CD8^+$ T cells by cytokines has been described during infection with other bacteria such as *Burkholderia pseudomallei* and *Listeria monocytogenes* (Lertmemongkolchai, Cai et al. 2001).

The development of a $CD8^+$ T cell response was analysed in two non-vaccinated animals after challenge with *M. bovis*. Similar to observations after BCG-vaccination, the mycobacteria-reactive cells were detected in the $CD8^{hi}TCR1^-CD45RO^+$ T cell subset and no response to mycobacteria was detected in the $CD8^{hi}TCR1^-CD45RO^-$ T cells.

The mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells detected after infection with *M. bovis* responded more highly to *M. bovis*-infected Mϕ than to BCG-infected Mϕ, indicating that these cells recognise antigen expressed by *M. bovis*, which are absent from or expressed at a low level by BCG-infected Mϕ. The kinetics of the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells response detected after infection with *M. bovis* differed between the two animals. In animal 496, mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells were initially detected at 6 wks post-infection, peaked at 8 wks and then declined to background levels by 11 wks post-infection. In animal 529 mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells were first detected at 8 wks post-infection and response of these cells increased by 11 wks post-infection. In order to obtain a clearer picture of the development of immune responses after infection a longer time course of infection is required. It is thought that peaks in the immune response may correlate with mycobacteria replication when mycobacterial antigens may be more abundant in the cytosol of infected cells.

The results of this study suggest that the use of the total $CD8^+$ population to investigate $CD8^+$ T cell response in bovine TB is unreliable and gives inconsistent results. The response detected in the total $CD8^+$ population was different to that detected in the $CD8^{hi}TCR1^-CD45RO^+$ T cells. The total $CD8^+$ cells from animal 529 responded to mycobacteria prior to infection, whereas the $CD8^{hi}TCR1^-CD45RO^+$ T cells did not. Additionally, post-challenge the total $CD8^+$ cells produced IFN-γ in response to uninfected and infected Mϕ. In contrast, no response was detected to uninfected Mϕ in the $CD8^{hi}TCR1^-CD45RO^+$ T cells. Although the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells are present albeit at a lower frequency in the total $CD8^+$ population, it expected that a similar, although smaller response of the $CD8^{hi}TCR1^-CD45RO^+$ T cells would be observed in the total $CD8^+$ cells. It is possible that a population of cells in the total $CD8^+$ cells are inhibiting the response of the $CD8^{hi}TCR1^-CD45RO^+$ T cells as a suppressive role for γδ T cells in bovine TB has been reported. It was demonstrated that depletion of γδ T cells in PBMC from infected animals enhanced proliferative responses to a range of mycobacterial antigens (Rhodes, Hewinson et al. 2001).

The two non-vaccinated animals infected with *M. bovis* were killed at 12 wks post-infection. Whereas animal 496 was found to have a relatively high lesion score typical of a non-vaccinated animal, suprisingly, animal 529 had a low lesion score, characteristic of a BCG-vaccinated animal. This suggests that the immune response induced by infection in animal 529 conferred a high degree of protection. The only difference observed between the immune responses of the two animals which may account for the variation in protection was that stronger responses in PBMC and $CD8^{hi}TCR1^-CD45RO^+$ T cells were detected at the end of the experiment in animal 529 compared to animal 496. It is thought that the more robust immune response elicited in animal 529 may have controlled the spread of the *M. bovis* and facilitated the clearance of the mycobacteria. This is in contrast with previous findings in cattle in which an increased production of IFN-γ by PBMC in response to ESAT-6 correlated with an increased lesion score (Vordermeier, Chambers et al. 2002). It is thought that in the protected vaccinated animals a robust immune response would be generated early during infection and then subside as the mycobacteria is cleared or contained. Whereas in the non-vaccinated animals, the development of the immune response may be

delayed and a prolonged heightened immune response would be observed as the mycobacteria replicate and disseminate. It is possible that analysis of T cell responses at the site of infection may provide a clearer picture of why animal 529 was protected and animal 496 was not.

These results demonstrate that there is animal to animal variation in the kinetics and magnitude of the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cell response elicited during *M. bovis* infection. The two animals used in this experiment were of unknown MHC haplotype and are likely to be different. The MHC haplotype of an animal determines the pattern of antigenic peptides expressed on MHC molecules therefore the mycobacterial peptides presented by the APC to T cells from each animal are likely to be different. It is thought that this heterogeneity between the animals may explain, in part, the difference in the pattern of responses observed and the levels of protection conferred.

Previously, $CD8^{+}$ cells have been proposed to contribute to the pathology of the disease in *M. bovis*-infected animals. It was reported that the transient depletion of $CD8^{+}$ cells within the first two weeks of infection resulted in animals having decreased lesion scores compared to control infected animals (Villarreal-Ramos, McAulay et al. 2003). However, the antibody used to deplete the $CD8^{+}$ cells will have depleted the $CD8^{+}$ NK cells, γδ T cells and αβ T cells. The results of this chapter show that the earliest point at which mycobacteria-reactive $CD8^{hi}TCR1^{-}$ T cells could be detected after infection with *M. bovis* was at 6 wks post-infection. Therefore, as the depletion of $CD8^{+}$ cells was carried out within the first two weeks of infection, it may not have strongly influenced the development of mycobacteria-reactive $CD8^{+}$ αβ T cells. It is more likely that the effects described in the depletion study are due to the removal of mycobacteria-reactive $CD8^{+}$ γδ T cells and $CD8^{+}$ NK cells that form part of the innate immune response induced by infection.

Vaccination of cattle with BCG has been shown to confer a significant degree of immunity to infection with *M. bovis*. However, it is clear that this immunity is not complete as other trials have reported that variable levels of protection were induced by BCG (Buddle, de Lisle et al. 1995; Buddle, Keen et al. 1995; Buddle, Wedlock et al. 2003; Hope, Thom et al. 2005). It is thought that different conditions of the trials may

have been a contributory factor to this observed variation. In addition, it has been proposed that exposure and sensitisation to environmental mycobacteria reduces the effectiveness of BCG (Buddle, Wards et al. 2002). Past studies of immune responses to infection and vaccination were evaluated in PBMC. This is the first study to follow the development of $CD8^+$ T cells responses induced by BCG-vaccination and the subsequent infection with *M. bovis*.

It was shown in chapter 5 that prior to infection, BCG vaccination of the three neonatal calves elicited mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells that responded to BCG-, *M. avium*- and *M. bovis*-infected Mϕ. In contrast, after infection with *M. bovis* mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells were detected only in animals 29 and 34 and responded mainly to *M. bovis*-infected Mϕ. In animal 23, although no response was detected in the $CD8^{hi}TCR1^-CD45RO^+$ T cells after infection with *M. bovis*, a strong proliferative response was observed in the PBMC indicating the induction of a robust $CD4^+$ T cell response.

In contrast to animal 23, the proliferative response detected in the PBMC from animals 29 and 34 after infection was weaker and less consistent than that of animal 23. These results suggest that infection with *M. bovis* induced a stronger $CD8^+$ T cell response in animals 29 and 34 while in animal 23 a stronger $CD4^+$ T cell response was induced.

At necropsy, all three animals were found to have a significantly lower lesion scores compared to the unvaccinated infected controls. This suggests that the strong response detected in the PBMC from animal 23 was protective. While in animals 29 and 34, the weaker and transient response of the PBMC may have been compensated for by the stronger protective responses of the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells in these animals.

Interestingly, prior to infection the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells in blood from the vaccinated animals responded to BCG-, *M. avium*- and *M. bovis*- infected Mϕ but after infection these cells responded predominantly to *M. bovis*-infected Mϕ. Therefore, $CD8^{hi}TCR1^-CD45RO^+$ T cells induced by BCG vaccination are likely to recognise an antigen that expressed by the three mycobacteria. Whilst the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells detected after infection recognise antigens that are specific to *M. bovis*. These results suggest that after infection with *M. bovis*, primary $CD8^+$ T cell responses are elicited in the BCG-vaccinated animals.

Similarly, $CD8^+$ T cells from TB patients respond to ESAT-6 and Ag85 whereas $CD8^+$ T cells from BCG-vaccinated individuals responded to Ag85 but not ESAT-6 (Smith and Dockrell 2000; Smith, Klein et al. 2000) . These findings suggest that after infection with *M. tuberculosis* or *M. bovis* in BCG-vaccinated individuals, primary $CD8^+$ T cell responses are elicited to *M. tuberculosis* or *M. bovis* specific antigens.

It is thought that the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells induced by BCG vaccination that responded to BCG and *M. avium* may have migrated into the infected tissues and are therefore not detected in blood after infection with *M. bovis*. It is also possible that these cells responded highly to infection at an earlier timepoint than when the first sample was taken at 5 wks post-challenge and subsequently underwent AICD.

BCG vaccination of cattle that have been pre-sensitised to mycobacteria by exposure to *M. avium* has been shown to result in an immune response directed against a shared antigen between BCG and *M. avium* (Howard, Kwong et al. 2002). Prior to vaccination, the three neonatal animals used in this experiment responded to BCG and *M. avium*-infected Mϕ. Therefore, it is possible that these animals had been exposed to *M. avium*. In agreement with previous findings, $CD8^{hi}TCR1^-CD45RO^+$ T cells induced by vaccination recognised antigens shared by BCG and *M. avium*. This skewing of the immune response in sensitised animals may partly explain why the $CD8^{hi}TCR1^-CD45RO^+$ T cells induced by BCG vaccination did not respond after infection with *M. bovis* as shared antigens may not be immunodominant during infection.

The control of mycobacterial replication is primarily determined by interactions between infected Mϕ and effector T cells. The culture of BCG- or *M. bovis*-infected Mϕ for 5 days, resulted in a reduction in the number of mycobacteria within the Mϕ. A greater decrease in BCG counts compared to *M. bovis* was observed, this suggests that *M. bovis* is better equipped to survive within Mϕ compared to BCG. It has been shown previously that *M. bovis* survives better than BCG in bovine monocyte-derived Mϕ as these Mϕ are permissive to the metabolism of *M. bovis* but not BCG (Liebana, Aranaz et al. 2000).

Previous studies in *M. bovis*-infected cattle, have used uracil uptake to investigate the effect of T cells on the metabolism of *M. bovis* and BCG within Mϕ. These studies have only measured the influence of T cells on the metabolic activity of the mycobacteria and don't provide information on numbers of mycobacteria. This is the first study to investigate the effect of $CD8^+$ T cells subsets from *M. bovis*-infected animals on mycobacterial numbers within Mϕ.

The results show that culture of infected Mϕ with $CD8^{hi}TCR1^-CD45RO^+$ T cells from both animals induced the largest decrease in BCG and *M. bovis* cfu. Surprisingly, the $CD8^{hi}TCR1^-CD45RO^-$ T cells also induced a reduction in bacterial numbers while in the other assays these cells did not display reactivity to *M. bovis*.

In contrast, the $CD8^{hi}TCR1^-CD45RO^+$ T cells from animal 34 proliferated and produced IFN-γ when cultured with *M. bovis* infected Mϕ. IFN-γ activates infected Mϕ to up-regulate RNI and ROI activity which can lead to the killing of mycobacteria (Denis 1991; Chan, Xing et al. 1992). Thereby, the production of IFN-γ may be a mechanism by which the $CD8^{hi}TCR1^-CD45RO^+$ T cells from animal 34 mediated the observed anti-mycobacterial activity. The mechanism by which the $CD8^{hi}TCR1^-CD45RO^+$ T cells from animal 23 and the $CD8^{hi}TCR1^-CD45RO^-$ T cells from both animals reduced mycobacterial counts is unclear, as these cells did not produce IFN-γ or proliferate after culture with infected Mϕ. Similar results have been reported, in which PBMC from both BCG-vaccinated and non-vaccinated animals reduced the metabolic rate of BCG and *M. bovis* inside Mϕ (Carpenter, Fray et al. 1997; Denis, Wedlock et al. 2004). It has been shown that the mechanisms of this mycobacteriostatic activity were different between the two groups of animals. The mycobacteriostatic activity observed in the PBMC from BCG-vaccinated animals was predominantly dependent upon production of IFN-γ and NO and did not require cell-contact. Whereas in the PBMC from non-vaccinated animals, the mycobacteriostatic activity was independent of IFN-γ and NO production and required cell contact (Denis, Wedlock et al. 2004). Likewise in *M. bovis*-infected animals, Liebana and co-workers reported that PBMC from uninfected and infected animals reduced the metabolic activity of *M. bovis* within Mϕ (Liebana, Aranaz et al. 2000).

In human studies, inhibition of mycobacterial replication was demonstrated in *M. tuberculosis*-infected Mϕ from PPD negative individuals and was mediated by both $CD4^+$ and $CD8^+$ T cells (Silver, Li et al. 1998). These results suggest that naïve T cells

can inhibit the metabolism of mycobacteria within infected Mϕ which may explain why culture with the $CD8^{hi}TCR1^{-}CD45RO^{-}$ T cells induced a decrease in mycobacterial numbers within Mϕ. Moreover, effector T cells, present in the sorted subsets, specific for an unrelated antigen may be acting to reduce mycobacterial numbers in a bystander manner and being activated by cytokines produced by the infected Mϕ.

Past studies reported that a greater inhibition of mycobacterial metabolism was induced by T cells from *M. bovis*-infected or BCG-vaccinated individuals compared to T cells from naïve individuals. Liebana and co-workers reported that PBMC from *M. bovis*-infected cattle induced a greater release of active mycobacteria from infected Mϕ compared to PBMC from uninfected animals. Furthermore $CD8^+$ T cell clones established from the infected animals induced a higher release of mycobacteria compared to $CD4^+$ T cell clones proposing a cytolytic function for the $CD8^+$ T cells (Liebana, Aranaz et al. 2000).

The mycobacteria-reactive $CD8^+$ T cells detected in this study may reduce mycobacterial counts by a number of different mechanisms. One mechanism may be the activation of infected Mϕ via production of cytokines such as IFN-γ. Another mechanism could be via the direct killing of the mycobacteria and the infected Mϕ through the release of cytotoxic granules, containing granulysin, perforin and granzymes, by the $CD8^+$ T cell. Worku et al demonstrated that T cells from vaccinated humans inhibited the growth and metabolism of BCG inside Mϕ and that these T cells simultaneously up-regulated expression of mRNA for IFN-γ, Granzyme A, granulysin and perforin (Worku and Hoft 2003). A third mechanism would be the killing of the infected Mϕ via interactions of membrane bound molecules such as FASL or TNF-α, which may result in the release of the active mycobacteria. The released mycobacteria could then be taken up and killed by an uninfected activated Mϕ or DCs. It is likely that a combination of these mechanisms mediate the inhibitory effect of T cells on mycobacterial growth and survival. Future experiments should be aimed at determining if a soluble factor is involved or if this inhibitory effect on the viability of mycobacteria requires cell-contact between T cells and infected Mϕ. It would also be interesting to investigate at which point post-infection do the $CD8^+$ T cells that exhibit this effect arise in the blood. In order to substantiate these findings additional control experiments are required these should include the analysis of the effect of $CD8^+$ T cells from uninfected animals on mycobacterial viability within Mϕ.

Vaccination with BCG is routinely administered subcutaneously while natural infection with either *M. tuberculosis* or *M. bovis* is thought to occur via the respiratory or gastrointestinal route. Most studies of immune responses in *M. bovis* infection of cattle analyse the reactivity of cells in PBMC or whole blood. One of the limiting factors of this study is that these experiments have been analysing the presence of mycobacteria-reactive $CD8^+$ T cells in blood. It is thought that to obtain a clearer understanding of immune responses induced by mycobacteria, future experiments should focus on immune mechanisms at the site of infection. In a preliminary experiment, cells from the BAL of three infected animals were stimulated overnight with BCG or *M. bovis* to investigate the presence of mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells in the lung. The results demonstrated the presence of IFN-γ expressing mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells in the BAL from the three infected animals. In murine studies, *M. tuberculosis*-specific $CD8^+$ T cells are recruited to the lung after infection and have been shown to be cytotoxic and lyse *M. tuberculosis*-infected Mϕ (Serbina, Liu et al. 2000).

These results implicate a role for $CD8^{hi}TCR1^-$ T cells in controlling the mycobacteria at the site of infection. Further analysis of these cells is required to investigate other effector functions and the kinetics of the appearance of these $CD8^{hi}TCR1^-$ T cells in the BAL.

To summarise, these results show that *M. bovis*-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells can be detected in blood after *M. bovis* infection of BCG-vaccinated and non-vaccinated animals. These cells are likely to play an important role in the control and containment of the mycobacteria as they were shown to proliferate, produce IFN-γ, and reduce mycobacterial numbers inside Mϕ. Although, the magnitude of the mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells responses were higher in the BCG-vaccinated animals compared to non-vaccinated animals, the mycobacteria-reactive $CD8^{hi}TCR1^-$ T cells induced by BCG vaccination were not detected in blood after infection.

CHAPTER 7: FINAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

7.1 Final Discussion

The increasing incidence of bovine TB in the UK demonstrates that the current control strategy, the test and slaughter policy, is insufficient. Improved control measures have been proposed to include the implementation of an effective vaccine and a more discriminatory diagnostic test. There are currently no licensed vaccines against bovine TB. BCG, the vaccine against human TB is not used in cattle as it interferes with the current diagnostic test and does not confer complete protection. The design of a more effective vaccine requires a greater understanding of both the immune response initiated by infection with *M. bovis*, the causative agent of bovine TB, and the protective immune response that is required for its clearance.

The importance of $CD8^+$ T cells in immunity to infection with *M. tuberculosis* was originally highlighted by studies in transgenic mice. (Flynn, Goldstein et al. 1992; Ladel, Daugelat et al. 1995). In humans, *M. tuberculosis*-specific $CD8^+$ T cells have been identified and proposed to play a critical role in the control of infection in healthy PPD-positive individuals. In cattle, mycobacteria-reactive $CD8^+$ T cells have been isolated from infected animals but the role of $CD8^+$ cells in immunity to *M. bovis* infection has not been characterised. Furthermore BCG vaccination of cattle induces a memory response that is able to control *M. bovis* infection and is thought to involve $CD4^+$ and $CD8^+$ T cells. Thus, the focus of this study is to investigate the kinetics of development and effector functions of memory mycobacteria-reactive $CD8^+$ T cells induced by BCG vaccination and/or *M. bovis* infection in cattle. It is thought that a greater understanding of the role of $CD8^+$ T cells in immunity to *M. bovis* infection in cattle may aid the design of vaccines that generate memory $CD8^+$ T cells with the capacity to facilitate clearance of *M. bovis*.

Past studies of $CD8^+$ T cell responses in cattle have used the total $CD8^+$ cell population which was shown in this study to contain $\alpha\beta$ ($CD3^+TCR1^-$) T cells, $\gamma\delta$ ($CD3^+TCR1^+$) T

cells and NK ($CD3^-TCR1^-$) cells. Furthermore this population of cells can be divided based upon level of expression of the CD8 molecule into hi and lo expressing cells. The $CD8^{hi}$ population contained mostly αβ and γδ T cells whilst the $CD8^{lo}$ population contained the majority of the NK cells. Thus the $CD8^{hi}TCR1^-$ T cells were isolated and further analysed in this study as these are mostly αβ T cells that form part of the adaptive immune response and develop memory T cell responses.

7.1.1 Differences in the proportion of memory $CD8^+$ T cells in blood with age and in different tissues

In order to investigate the development of memory responses to mycobacteria in cattle, the initial experiments were aimed at defining naïve and memory $CD8^+$ T cells. The markers used in these experiments were selected from those used previously to define memory $CD8^+$ T cell in human studies. By determining the surface phenotype of a T cell it is possible to attain some insight into the history of that particular cell. It is also understood that these results should not be over-interpreted as T cells that express the same phenotype do not necessarily express the same function. Therefore in this study surface phenotype and effector molecule expression were analysed simultaneously on $CD8^{hi}TCR1^-$ T cells in blood, LN and BAL.

In humans, changes in the composition of the circulating T cell compartment occur during ageing that result in aged individuals having an increased proportion of effector and effector memory and a decreased proportion of naïve T cells (Fagnoni, Vescovini et al. 2000; Hong, Dan et al. 2004). Similar age-associated changes were observed in cattle with an increase in the proportion of primed/memory $CD8^{hi}TCR1^-$ T cells in blood as shown by an increase in the percentage of cells that are $CD45RO^+$, $CD25^+$, $CD26^+$, $CD45RA^-$, $CD62L^-$, $CD28^-$ and $CD27^-$. This increase in the proportion of memory $CD8^{hi}TCR1^-$ T cells with age indicates that older cattle may have a diminished ability to respond to new infections as in the 5-10yr group up to 40% of the $CD8^{hi}TCR1^-$ T cells are $CD45RO^+$. The observed increase in cells with an effector/memory phenotype corresponded with an increase in the percentage of $CD8^{hi}TCR1^-$ T cells that constitutively express perforin and IFN-γ after brief mitogen stimulation.

Consistently a higher percentage of $CD8^{hi}TCR1^-$ T cells expressed IFN-γ compared to perforin and of these cells a higher percentage expressed CD28 compared to $perforin^+CD8^{hi}TCR1^-$ T cells which were mostly $CD28^-$. This suggests that $perforin^+$ cells are more differentiated than those that express IFN-γ alone and that acquisition of perforin expression may occur further along the pathway of differentiation of effector T cells.

The surface phenotype of $CD8^{hi}TCR1^-$ T cells present in the BAL was analysed as it is thought that only activated/memory T cells express the appropriate surface molecules to facilitate migration into peripheral tissues such as the lung. Furthermore as the lung is thought to be the primary site of TB infection an increased understanding of the populations of cells present in this tissue may aid the design of vaccines that target mycobacterial specific T cells to the lung. The $CD8^{hi}TCR1^-$ T cells in the BAL expressed an activated/memory phenotype with greater than 95% of the cells being $CD45RO^+$. A high proportion of these cells also expressed the activation marker CD25 indicating that these cells are poised to respond quickly to infection. It is also possible that these cells can contribute to the clearance of unrelated antigens by responding in a bystander manner to IL-2 produced by other responding T cells. CD25 is also known to be expressed by a subset of regulatory T cells that act to dampen down immune responses and may play an important role in the airways. However a high proportion of the $CD8^{hi}TCR1^-CD25^+$ T cells express IFN-γ and perforin, therefore it remains to be investigated whether this population of cells contains suppressor $CD8^+$ T cells.
Minor populations of $CD8^{hi}TCR1^-$ T cells were also present in the BAL that may represent cells at different stages of differentiation or different effector cell populations as in human studies different viral pathogens were shown to induce effector $CD8^+$ T cells with different surface phenotypes (Appay, Dunbar et al. 2002). Interestingly, a small population of $CD8^{hi}TCR1^-CD45RO^-CD45RA^+$ T cells was identified in the BAL that may represent either effector cells that have yet to become memory T cells or a population of memory T cells that have re-expressed CD45RA. In humans these CD45RA-primed cells arise during chronic viral infections and are often associated with CMV seroconversion (Appay, Dunbar et al. 2002; Kuijpers, Vossen et al. 2003).

In contrast to the BAL, the population of $CD8^{hi}TCR1^-$ T cells in the LN predominantly expressed a naïve phenotype. It was demonstrated that a small population of memory cells are also present in bovine LN. This memory population was found to be $CD8^{hi}TCR1^-CD45RO^+CD62L^+$, which correspond to central memory T cells described in mice as being primed cells that recirculate through LN. These central memory T cells in bovine LN expressed perforin and IFN-γ whereas in mice the effector functions of these cells are controversial, as originally they were shown to have limited effector functions, while others have shown that they produce IFN-γ and are cytotoxic (Sallusto, Lenig et al. 1999; Wherry, Teichgraber et al. 2003).

The increased percentage of $CD8^{hi}TCR1^-CD45RO^+$ T cells in the BAL compared to LN was also reflected in an increase in the percentage of BAL cells expressing perforin and IFN-γ.

Interestingly, although the majority of cells in the BAL appear to be memory/effector T cells a similar percentage of $CD8^{hi}TCR1^-CD28^+$ T cells were present in both the BAL and LN. In addition, compared to the LN a higher percentage of $CD8^{hi}TCR1^-$ in the BAL had lost expression of CD27. Expression of CD27 is thought to be lost after repeated antigen stimulation whereas loss of CD28 is associated with replicative senescence. Therefore these results suggest the memory $CD8^{hi}TCR1^-$ T cells in the BAL may have undergone multiple rounds of stimulation but have not become senescent (Kuijpers, Vossen et al. 2003; Baars, Sierro et al. 2005).

Interestingly, similar to the age-associated increase in the percentage of circulating $CD8^+$ T cells that express an activated/memory phenotype, infection with *M. bovis* also induced an increase in the proportion of total $CD8^+$ cells that were $CD45RO^+$, $CD25^+$ and $CD45RA^-$. It is likely that these activated/memory T cells increase in blood after infection with *M. bovis* because they are being recruited to the site of infection. It is possible that they are enroute to the lungs as a high proportion of the $CD8^+$ T cells in the BAL were found to be $CD45RO^+$, $CD45RA^-$ and $CD25^+$. Significant increases in the number of activated circulating $CD8^+$ T cells are also observed during infection with Epstein Barr virus (EBV) with up 44% of total $CD8^+$ T cell population being specific for EBV epitopes (Callan, Tan et al. 1998).

One limitation of the phenotyping studies is that only differences in the percentages of cells were analysed. To provide a fuller picture of cellular changes during infection or activation differences in absolute numbers of cells should also be investigated.

7.1.2 BCG vaccination generates memory $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells

From the initial phenotyping experiments it was found that expression of CD45RO on $CD8^{hi}TCR1^{-}$ T cells defined a population of cells that express effector molecules and have specific migratory capacities and are likely to be memory T cells. Moreover previous studies in humans and mice have shown that the two main populations of memory cells, central and effector both express CD45RO. Therefore to study the development of memory $CD8^{+}$ T cell response in cattle, the $CD8^{hi}TCR1^{-}$ T cells were sorted into $CD45RO^{+}$ and $CD45RO^{-}$ T cells.

BCG vaccination of 6 mth old animals generated BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells. These cells mediated recall responses to BCG-infected Mϕ as detected by proliferation, production of IFN-γ, up-regulation of perforin expression and cytotoxicity. It is thought that these $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells could contribute to the control of mycobacteria via a combination of these effector functions. Similarly in healthy BCG vaccinated humans, BCG-specific memory $CD8^{+}$ T cells stimulated *in vitro* with BCG produced IFN-γ, up-regulated perforin expression and were cytolytic (Turner and Dockrell 1996; Smith, Malin et al. 1999). It was shown that the BCG-specific memory $CD8^{+}$ T cells recognised BCG in an MHC class I restricted manner, as blocking of HLA-A, B or C abrogated the response.

The restriction of the responding $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells in BCG-vaccinated animals has yet to be investigated. However, it is likely that the response of $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells measured in this study is not CD1-restricted as the Mϕs used as APC in these experiments have been shown not to express CD1 on their cell surface and expression of CD1 is largely confined to DCs (Stenger, Niazi et al. 1998).

The observed proliferative capacity of the BCG-reactive memory $CD8^{hi}TCR1^{-}$ $CD45RO^{+}$ T cells after *in vitro* stimulation implies that during infection, these cells could be activated to generate mycobactericidal effector cells. The proliferative potential of these cells indicates that they are similar to central memory T cells that

display an increased capacity to proliferate in response to antigen compared to effector memory T cells. For this reason the former have been proposed to be more efficient at mediating protective immunity (Wherry, Teichgraber et al. 2003).

The mycobacteria reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells induced by BCG vaccination produced high levels of IFN-γ in response to stimulation with BCG-infected Mϕ. The frequency of these IFN-γ producing cells was not analysed in these experiments. *Ex vivo* ELISPOTs were performed to determine this, however, an extremely high number of spots was observed in response to uninfected Mϕ. This high background response may not occur in a cultured ELISPOT, which would be more similar to the 5 day proliferation assay already set-up.

The importance of IFN-γ in the control of mycobacterial infections has been demonstrated by an increased susceptibility to infection of IFN-γ receptor KO mice (Cooper, Dalton et al. 1993; Flynn, Chan et al. 1993) and in humans with genetic defects in the ability to produce or respond to IFN-γ (Jouanguy, Altare et al. 1997; Dorman and Holland 1998; Jouanguy, Lamhamedi-Cherradi et al. 1999; Dupuis, Doffinger et al. 2000). IFN-γ activates microbicidal functions of Mϕ in part, through the production of reactive oxygen and nitrogen intermediates (Nathan, Prendergast et al. 1984). Exposure of microbes to these molecules leads to an accumulation in protein, lipid and DNA damage and the subsequent death of the microbe (Nathan and Shiloh 2000). Virulent mycobacteria however have evolved strategies to evade the microbicidal functions of Mϕ through the production of proteins that detoxify reactive species (Sherman 1995) and the inhibition of macrophage responsiveness to IFN-γ (Ting, Kim et al. 1999; Ehrt, Schnappinger et al. 2001; Kincaid and Ernst 2003; Fortune, Solache et al. 2004). Although infected Mϕ have a reduced capacity to respond to IFN-γ, Mϕ activated with IFN-γ prior to infection display increased mycobactericidal function (Chan, Xing et al. 1992; Ehrt, Schnappinger et al. 2001). Thereby the IFN-γ produced by the $CD8^{hi}TCR1^{-}CD45RO^{+}$T cell may function during infection with *M. bovis* to activate uninfected Mϕ prior to the uptake of mycobacteria. These activated Mϕ may in turn take up and kill mycobacteria that have been released from lysed Mϕ or from the uptake of apoptotic blebs containing mycobacteria.

Similar to studies in BCG-vaccinated humans, the memory $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells induced by BCG vaccination of cattle up-regulated expression of perforin after *in vitro* stimulation with BCG (Smith, Malin et al. 1999; Tsunetsugu-Yokota, Tamura et al. 2002). The responding $CD8^{hi}TCR1^{-}perforin^{+}$ T cells expressed CD45RO but lacked expression of CD62L and CD28. Again this is comparable to BCG-vaccinated humans in whom the BCG-specific $CD8^{+}$ T cells that up-regulated perforin expression were $CD45RA^{-}$ (Tsunetsugu-Yokota, Tamura et al. 2002). In two of the vaccinated animals a small percentage of $CD8^{hi}TCR1^{-}perforin^{+}$ T cells were $CD62L^{+}$ and $CD28^{+}$ suggesting that the responding memory T cells may have divided and generated both effector memory cells and central memory T cells.

Surprisingly expression of perforin did not directly correlate with lysis of infected Mϕ as the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from only two out of the three animals that up-regulated perforin, lysed BCG-infected Mϕ. The effect of these BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells on actual mycobacterial numbers remains to be determined. It is thought that lysis of infected Mϕ by these $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells may result in a reduction of mycobacterial numbers via the uptake of released mycobacteria by uninfected Mϕ that have been activated by IFN-γ. In humans, *M. tuberculosis* specific $CD8^{+}$ T cells were found to restrict mycobacterial growth inside Mϕ via a perforin and FASL independent mechanism (Canaday, Wilkinson et al. 2001). The identification of the mycobactericidal granulysin, which was shown to be expressed by activated $CD8^{+}$, $CD4^{+}$, γδ and NK cells, may provide a means by which *M. tuberculosis* specific $CD8^{+}$ T cells inhibit the growth of *M. tuberculosis* . In these experiments only the BCG-reactive $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from one animal expressed the mRNA for granulysin and this expression required stimulation with BCG. It is thought that these $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells are likely be more effective at controlling mycobacterial infections compared to the cells that express only perforin, as active peptides of granulysin have been shown to kill mycobacteria (Stenger, Hanson et al. 1998; Endsley, Furrer et al. 2004). It has been proposed that perforin and granulysin are contained within the same cytotoxic granule as the killing of intracellular mycobacteria by granulysin requires the assistance of pore-forming properties of perforin (Stenger, Hanson et al. 1998).

The capacity of *M. tuberculosis*-specific $CD8^+$ T cells from humans and mice to reduce viability of *M. tuberculosis* via a mechanism that is independent of granule exocytosis and FASL suggests other pathways are involved. The binding of other cell surface molecules such as members of the TNF-receptor family and the production of ATP by $CD8^+$ T cells has been shown to induce apoptosis of the infected Mϕ and may lead to the uptake and killing of the mycobacteria by proficient uninfected Mϕ.

The mechanism by which the BCG-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells described in this study induced lysis of the BCG-infected Mϕ requires further investigation as it may involve the interaction of several mechanisms. It would be useful to try blocking experiments using degranulation of the T cells by prior treatment with strontium chloride to exclude granule exocytosis or incorporate antibodies to block FAS-FASL or membrane bound TNF-α interactions. Furthermore it would be of interest to determine whether lysis of BCG-infected Mϕ affects the viability of the mycobacteria.

The results presented in this study demonstrate the requirement for sorting the $CD8^+$ cells present in bovine PBMC as the responses measured to either vaccination or infection in the total $CD8^+$ population were very different to those measured in the $CD8^{hi}TCR1^-CD45RO^+$ T cell population. In the 5-day proliferation assay, cells within the total $CD8^+$ population frequently responded to uninfected Mϕ whereas no response to uninfected Mϕ was detected in the $CD8^{hi}TCR1^-CD45RO^+$ T cells. The antigens recognised by the total $CD8^+$ population and the $CD8^{hi}TCR1^-CD45RO^+$ T cells also differed. The total $CD8^+$ T cell responded to PPD-B whereas no response to this antigen was observed in the $CD8^{hi}TCR1^-CD45RO^+$ T cells. Furthermore the total $CD8^+$ T cells also repeatedly responded to mycobacteria prior to vaccination or infection whereas only in neonatal animals did the $CD8^{hi}TCR1^-CD45RO^+$ T cells respond prior to vaccination. These observations indicate that different subtypes of T cells are responding in the two populations and that the study of memory $CD8^+$ αβ T cell responses in cattle requires sorting of $CD8^{hi}TCR1^-CD45RO^+$ T cells from the total $CD8^+$ population.

7.1.3 A prime-boost strategy to enhance the protection afforded by BCG

Although vaccination with BCG was found to induce $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells that exhibit effector functions, it still remains that the immune response induced by BCG-vaccination is not adequate to clear *M. bovis* or *M. tuberculosis* infection. To date no other vaccine has provided a greater level of protection than BCG, therefore complete replacement of this vaccine would be unlikely in the near future. A more achievable aim would be to boost the response induced by BCG using a vaccine that delivers mycobacterial antigens that induce robust $CD4^{+}$ and $CD8^{+}$ T cell responses. One such antigen is Ag85 which is composed of three subunits A, B and C. Both CD4 and CD8 T cell epitopes have been identified in the Ag85A subunit and DC expressing peptides from this molecule have been shown to induce strong $CD4^{+}$ and $CD8^{+}$ T cell responses in mice (McShane, Behboudi et al. 2002). The boosting of BCG with the recombinant viral vector, MVA expressing Ag85A, is currently in phase II clinical trials and preliminary data shows that this considerably boosts the immune responses to PPD-B and Ag85A peptides (McShane, Pathan et al. 2004; McShane, Pathan et al. 2005).

In this study a prime-boost strategy involving priming with vaccinia virus expressing Ag85A and boosting with BCG was assessed for its capacity to induce strong $CD8^{+}$ T cell responses against Ag85A. VV-Ag85 induced a strong response in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells and the subsequent administration of BCG considerably boosted this response. Surprisingly, at 4 wks post-BCG no responding $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells could be detected in blood. One possibility is that the responding cells have migrated into the tissues. However at 8 wks post-BCG the responding $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells were again detected in blood. Although these results are preliminary they demonstrate that it is possible to boost the $CD8^{+}$ T cell responses to mycobacteria in cattle using a heterologous prime-boost vaccine strategy. In the future more systematic experiments should be performed comparing immune responses induced by VV-Ag85 prime and BCG boost or BCG prime and VV-Ag85 boost, with BCG alone.

7.1.4 Differences between $CD8^+$ T cell responses induced by *M. bovis* infection of BCG vaccinated and non-vaccinated animals

The comparison of immune responses induced by *M. bovis* infection of non-vaccinated animals that develop severe disease and BCG-vaccinated animals that are able to contain the infection may provide insights into protective immune responses. *M. bovis* infection induced mycobacterial reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells in both vaccinated and non-vaccinated animals, these cells proliferated and produced IFN-γ. The main difference observed between the two groups of animals was the magnitude of the $CD8^{hi}TCR1^-CD45RO^+$ T cell response. After infection with *M. bovis*, the $CD8^{hi}TCR1^-CD45RO^+$ T cells from BCG-vaccinated proliferated more highly and produced higher levels of IFN-γ in response to *M. bovis*-infected Mϕ compared to cells from non-vaccinated animals. The greater response of the $CD8^{hi}TCR1^-CD45RO^+$ T cells from the BCG-vaccinated animals may be due to an increased frequency of mycobacteria-reactive T cells at the time of infection. Moreover it is also possible that the *M. bovis*-reactive $CD8^+$ T cells induced by infection of the BCG-vaccinated animals have a greater capacity to express effector functions due to the provision of help by the mycobacteria-reactive memory $CD4^+$ T cells that are likely to be present in the BCG-vaccinated animals but not in the non-vaccinated animals at the time of infection with *M. bovis*. In mice, it has been reported that although $CD4^+$ T cell help is not required for the development of primary $CD8^+$ T cell responses, the generation of functional memory $CD8^+$ T cells is reduced in the absence of $CD4^+$ T cells (Bourgeois and Tanchot 2003; Janssen, Lemmens et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003). Furthermore, it is likely that the level of killing of mycobacteria-infected Mϕ is greater in the BCG-vaccinated animals due to the higher frequency of mycobacteria-reactive T cells. This may result in an increased presentation of mycobacterial antigens on MHC class I molecules as it has been shown that uptake of apoptotic bodies containing mycobacteria by APC can lead to the presentation of mycobacterial antigens on MHC class I molecules (Winau, Weber et al. 2006).

In the non-vaccinated animals that were infected with *M. bovis*, stronger responses to mycobacteria developed in the PBMC compared to the $CD8^{hi}TCR1^-CD45RO^+$ T cells suggesting that the response induced by infection was predominantly mediated by

$CD4^+$ T cells. Conversely in two out of the three BCG-vaccinated animals the response of $CD8^{hi}TCR1^-CD45RO^+$ T cells was greater than that of the PBMC. In the remaining BCG-vaccinated animal the response in the PBMC was much higher than that of the other two vaccinated animals and no response was detected in the $CD8^{hi}TCR1^-CD45RO^+$ T cells from this animal. The protection data showed that one of the non-vaccinated animals and the three BCG-vaccinated animals were partially protected containing fewer and smaller lesions with only one or two tissues being affected. The other non-vaccinated animal 496, had more severe disease with multiple necrotic lesions present in a number of tissues.

The only difference observed between the two non-vaccinated infected animals that may account for the difference in protection, is that the $CD8^{hi}TCR1^-CD45RO^+$ T cell response in the protected animal was much stronger at the end of the experiment than that in the animal that had a more severe disease. It is has been shown in mice that whereas $CD4^+$ T cells may be more effective at controlling mycobacterial infections during the early stages of infection $CD8^+$ T cells were significantly more effective than $CD4^+$ T cells at controlling *M. tuberculosis* infection during latency (van Pinxteren, Cassidy et al. 2000).

The observed difference may also be related to genetic differences between the animals and this may influence the effectiveness of the immune response in containing mycobacterial infections. The induction of an immune response to mycobacterial antigens is dependent upon the epitopes expressed on MHC molecules. As the MHC region is highly polymorphic, genetic variation in expression of particular MHC haplotypes accounts for the ability or inability of individuals to respond to specific antigens. If the response to a specific antigen is critical in immunity to the mycobacteria then, those animals unable to make a response may not control the infection.

The three BCG-vaccinated animals showed similar degrees of protection even though differences were observed in the immune response elicited to infection. It was demonstrated that the PBMC response in the two animals that developed mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells was lower than that observed in the animal in which no $CD8^{hi}TCR1^-CD45RO^+$ T cell response could be detected. Given that the main population of responding cells in the PBMC is likely to be $CD4^+$ T cells, these results suggest that some redundancy may exist between the functions of $CD4^+$ T cells and

$CD8^+$ T cells induced during *M. bovis* infection in cattle. In mice deficient in $CD4^+$ T cells an increased number of IFN-γ producing $CD8^+$ T cells were observed in the lungs following aerosol challenge with *M. tuberculosis* (Caruso, Serbina et al. 1999; Serbina and Flynn 2001). It has been shown that BCG vaccination of mice deficient in $CD4^+$ T cells provided protection against *M. tuberculosis* infection when the mice were infected at either 6 or 12 wks post-vaccination but not in animals infected at 3 wks post-vaccination (Wang, Santosuosso et al. 2004). These results indicate that in the absence of $CD4^+$ T cells, the BCG-specific $CD8^+$ T cells can provide a level of protection against infection. Therefore, in animals 29 and 34 that generated a strong $CD8^+$ T cell response but weak PBMC responses, the $CD8^+$ T cell may have played a more prominent role in controlling the *M. bovis* infection. In animal 23, no mycobacteria-reactive $CD8^+$ T cells could be detected in blood post-infection, a strong response was detected in the PBMC, therefore in this animal the protection against infection may have been afforded by mycobacteria-reactive $CD4^+$ T cells.

7.1.5 Fate of memory $CD8^{hi}TCR1^-CD45RO^+$ T cells induced by BCG vaccination after infection with *M. bovis*

Sequestration of BCG-induced $CD8^{hi}TCR1^-CD45RO^+$ T cells in infected tissues

One of the most confusing observations of this study is that the $CD8^{hi}TCR1^-CD45RO^+$ T cells induced by BCG-vaccination respond to BCG-, *M. avium*- and *M. bovis*-infected Mϕ prior to infection while post-infection the $CD8^{hi}TCR1^-CD45RO^+$ T cell present in blood only respond to *M. bovis* infected Mϕ.

Even if the $CD8^{hi}TCR1^-CD45RO^+$ T cells induced by BCG vaccination are not activated during infection with *M. bovis* it might have been expected that they would still be detectable in blood. However it appears that in response to infection with *M. bovis* either the antigen specificity of these cells has changed or more likely their frequency in blood has declined due to the recruitment of these memory cells to the infected tissues during the early stages of infection. A recent study showed that more IFN-γ-producing T cells were present in granulomas formed in response to *M. bovis* infection of BCG vaccinated animals compared to non-vaccinated animals. In addition, the granulomas observed in BCG-vaccinated animals showed a reduced degree of necrosis and fibrosis and contained less acid-fast mycobacteria, compared to those

formed in the non-vaccinated animals (Johnson, Gough et al. 2006). Sequestration of mycobacteria-specific T cells at the site of infection has been shown to occur in TB patients with active disease (Dieli, Friscia et al. 1999). Studies in children with active TB reported that after chemotherapy, an increase in the percentage of Ag85A-specific $CD8^+$ T cells expressing an effector phenotype ($CD45RA^+CCR7^-$) and a decrease in cells expressing a central memory phenotype ($CD45RA^-CCR7^+$) was observed in blood. It is possible that the observed increase in effector Ag85A-specific $CD8^+$ T cells in blood after chemotherapy because during active disease these cells are sequestered at the site of infection and upon resolution of infection are released back into the circulation. In support of this hypothesis, the Ag85A-specific $CD8^+$ T cells present in the cerebral spinal fluid of one child with TB meningitis were mostly effector cells ($CD45RA^+CCR7^-$) whereas in the blood they were largely central memory cells ($CD45RA^-CCR7^+$) (Caccamo, Meraviglia et al. 2006). These findings support the hypothesis that the memory $CD8^{hi}TCR1^-CD45RO^+$ T cells induced by BCG-vaccination have been activated during the early stages of *M. bovis* infection and were subsequently recruited into the infected tissues.

Exhaustion of BCG-induced memory $CD8^+$ T cells

It is also possible that the memory mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells induced by BCG vaccination prior to challenge with *M. bovis* may have become anergic or exhausted and therefore are unable to respond *in vitro*. In chronic infections, the host immune response is unable to clear the pathogen. It is known that the functional effector $CD8^+$ T cells generated in the early stages of infection gradually lose function during the course of persistent infections. Exhaustion of Ag-specific $CD8^+$ T cells was first described in persistent LCMV infection of mice and subsequently was shown to occur during infection with HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV) in humans (Gallimore, Glithero et al. 1998; Zajac, Blattman et al. 1998; Letvin and Walker 2003; Pantaleo and Koup 2004; Rehermann and Nascimbeni 2005).

Thus, the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells may have become exhausted by repetitive stimulation with mycobacteria. Prior to vaccination with BCG these cells responded highly to both BCG and *M. avium* infected Mϕ, suggesting exposure to environmental mycobacteria. The subsequent vaccination with BCG

boosted the response of the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells and then inoculation with *M. bovis* is likely to have further stimulated these T cells. Recently, in mice chronically infected with LCMV, it was shown that by blocking the PD-1 receptor on exhausted virus-specific $CD8^{+}$ T cells, this restored their ability to exhibit effector functions (Barber, Wherry et al. 2006). Therefore, this could have been performed on the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from the *M. bovis* infected, BCG-vaccinated animals. However, the lack of a response in the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells to BCG and *M. avium* post-challenge with *M. bovis* is unlikely to be due to anergy or exhaustion as the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells from non-vaccinated did not response to mycobacteria prior to infection and also responded more highly to *M. bovis* infected Mϕ rather than BCG-infected Mϕ.

7.1.6 Differences in antigen specificity of $CD8^{+}$ T cells induced by *M. bovis* infection and BCG-vaccination

One of the reasons that the $CD8^{+}$ T cells induced by BCG-vaccination fail to provide complete protection against *M. bovis* infection may be that a number of antigens are likely to be expressed during *M. bovis* infection that will not be expressed after vaccination with BCG. Furthermore the memory $CD8^{+}$ T cells induced by BCG may not recognise protective antigens expressed during *M. bovis* infection. The $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells induced by BCG vaccination of the neonatal animals responded to BCG-, *M. avium*- and *M. bovis*-infected Mϕ, it is therefore likely that these cells are recognising antigens that are conserved between the three species of mycobacteria. Similarly, $CD8^{+}$ T cells from BCG-vaccinated humans recognise and respond to BCG-, *M. avium*- and *M. tuberculosis*-infected target cells (Esin, Batoni et al. 1996; Smith, Malin et al. 1999).

After infection with *M. bovis*, the $CD8^{hi}TCR1^{-}CD45RO^{+}$ T cells present in blood from both the vaccinated and non-vaccinated animals responded to *M. bovis* infected Mϕ and not to BCG-infected Mϕ. This suggests primary $CD8^{+}$ T cells responses are induced by infection with *M. bovis* that are directed towards antigens expressed by *M. bovis* but absent from BCG and *M. avium*. Previously, reactivity to ESAT-6 was found to differentiate between infected and vaccinated cattle (Buddle, Parlane et al. 1999). ESAT-6 is encoded by the RD1 region of *M. bovis* which is absent from BCG. More

recently, it was shown that a small percentage of $CD8^+$ cells from animals naturally infected with *M. bovis* were stimulated to express IFN-γ after culture with ESAT-6 16-mer peptides (Vitale, Reale et al. 2006), suggesting that ESAT-6 may be presented to $CD8^+$ T cells during natural and experimental *M. bovis* infection of cattle. Studies in humans have shown that ESAT-6 specific $CD8^+$ T cells are generated during *M. tuberculosis* infection but are not present in BCG-vaccinated subjects. In addition higher responses to ESAT-6 were demonstrated in TB patients after antibiotic treatment (Vekemans, Lienhardt et al. 2001).

Moreover, vaccination with a fusion protein incorporating ESAT-6 and Ag85B was found to be protective against *M. tuberculosis* infection in guinea pigs and macaques (Olsen, Williams et al. 2004; Langermans, Doherty et al. 2005). These finding suggest that immune responses directed against ESAT-6 are protective which may reflect the fact that this antigen is expressed during the early stages of infection and therefore will elicit mycobacteria-reactive effector T cells prior to establishment of chronic infection. Although ESAT-6 is a potential vaccine candidate that could be used in combination with BCG, is it unlikely that it will be used in vaccines against bovine TB as it been proposed to be incorporated into diagnostic tests and immune responses to this antigen have been shown in some studies to correlate with disease severity while others have shown that animals with severe lesions did not respond to this antigen (Vordermeier, Chambers et al. 2002; Hope, Thom et al. 2005). Further work is required to determine the antigen specificity of T cell responses elicited by *M. bovis* infection in protected and non-protected animals.

7.1.7 Relationship between $CD8^+$ T cell responses to mycobacteria observed *in vitro* and *in vivo*

The data presented in this thesis represents experiments that were largely performed with cells isolated from blood of BCG-vaccinated and/or *M. bovis*-infected animals. Although the study of cells present in blood can provide an idea of the capacity of these cells to react to antigen and exhibit effector functions *in vivo*, they fail to illustrate what is actually occurring at the site of infection or whether these cells can actually mediate recall responses *in vivo*. This is a major limitation of this study and therefore the ability of memory $CD8^+$ T cells, induced by BCG vaccination, to mediate secondary response

to mycobacteria *in vivo* was analysed. In this preliminary experiment, BCG-vaccinated animals were either inoculated intradermally with BCG or PBS and 3-4 days later the draining lymph node was removed. $CD8^+CD3^+Perforin^+$ T cells were present in the draining LN from BCG-vaccinated animals challenged with BCG which were not observed in the vaccinated animals challenged with PBS. This suggests that the BCG-reactive $CD8^+$ T cells induced by BCG vaccination of non-sensitised animals are able to mount secondary responses to mycobacteria *in vitro* and *in vivo*.

In *M. bovis* infected animals irrespective of vaccination status the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells detected in blood respond to *M. bovis* infected Mϕ but not to BCG-infected Mϕ. Conversely, $CD8^{hi}TCR1^-$ T cells in the lungs of non-vaccinated *M. bovis* infected animals responded to overnight stimulation with either BCG or *M. bovis*. This suggests that the population of mycobacteria reactive $CD8^+$ T cells present in the blood may not be representative of those present at the site of infection. This observation supports the hypothesis that the mycobacteria-reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells present in blood from BCG-vaccinated animals prior to infection with *M. bovis* and not detected in blood post-infection, are likely to have been recruited into infected tissues.

Studies in mice have shown that mycobacteria-reactive memory $CD8^+$ T cells are recruited into the lung during *M. tuberculosis* infection (Serbina and Flynn 2001). In cattle, an increased number of IFN-γ expressing cells were observed in granulomas from BCG-vaccinated *M. bovis* infected animals compared to non-vaccinated infected animals (Johnson, Gough et al. 2006).

7.2 Conclusions

In summary, different subsets of $CD8^+$ T cells have been identified in cattle by expression of surface and effector molecules and anatomical location. Furthermore age-related changes occur in the composition of the $CD8^{hi}TCR1^-$ T cell population in blood with an increase in primed/memory T cells and a decrease in naïve T cells. These findings may have important implications for the design of vaccine strategies in cattle as they indicate that vaccination may not be as effective in older animals compared to younger animals due to the decrease in naïve T cells. A population of memory T cells were identified in the LN that is analogous to central memory T cells. Furthermore a population of $CD8^{hi}TCR1^-CD45RA^+$ T cells that express effector molecules analogous to CD45RA-primed cells described in human studies was also identified in cattle.

The results of this study suggest that BCG vaccination induces functional memory $CD8^{hi}TCR1^-CD45RO^+$ T cells that mediate recall responses *in vitro*. These cells were shown to proliferate, produce IFN-γ, up-regulate perforin expression and kill BCG-infected Mϕ in response to in vitro stimulation with live mycobacteria. The fate of these cells after infection with *M. bovis* is unclear but they may be sequestered at the site of infection.

M. bovis reactive $CD8^{hi}TCR1^-CD45RO^+$ T cells develop in response to *M. bovis* infection in cattle. In blood, these cells recognise antigens that are specific to *M. bovis* whereas those found at the site of infection recognise antigens expressed by both BCG and *M. bovis*. Effector functions of these cells include proliferation, production of IFN-γ and inhibition of mycobacterial numbers inside Mϕ.

This study provides a model to investigate the efficacy of future vaccines or vaccination strategies at inducing $CD8^+$ T cells responses and protection against *M. bovis* infection in cattle.

7.3 Future Work

Future studies should be aimed at defining the antigen specificity of the mycobacteria-reactive $CD8^+$ T cells that are generated in response to vaccination with BCG and/or infection with *M. bovis*. The results of this study indicate that BCG-vaccination induces $CD8^+$ T cells that respond to antigens that are highly conserved between BCG, *M. avium* and *M. bovis*. Whilst *M. bovis* infection induces $CD8^+$ T cells in blood that respond to antigens specific to *M. bovis* that are absent from BCG and *M. avium*. Future studies should also investigate whether differences in antigen specificity are related to differences in the effector function and migratory behaviour of mycobacteria-reactive $CD8^+$ T cells.

The results of this study demonstrate the importance of analysing response against mycobacteria in blood and at the site of infection. Therefore future studies of $CD8^+$ T cell response induced by mycobacteria should compare and investigate the relationship between the $CD8^+$ T cells in blood and at the site of infection.

It was shown that BCG vaccination induces functional memory $CD8^+$ T cells that are likely to contribute to containment of *M. bovis*; however the memory immune response induced by BCG fails to eradicate the mycobacteria. The administration of a booster vaccine may enhance this memory $CD8^+$ T cell response and this study provides the basis on which to investigate this prospect.

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